# LAB BOOK

## Tolerance Maximisation

# Week 1

**Tasks completed:**

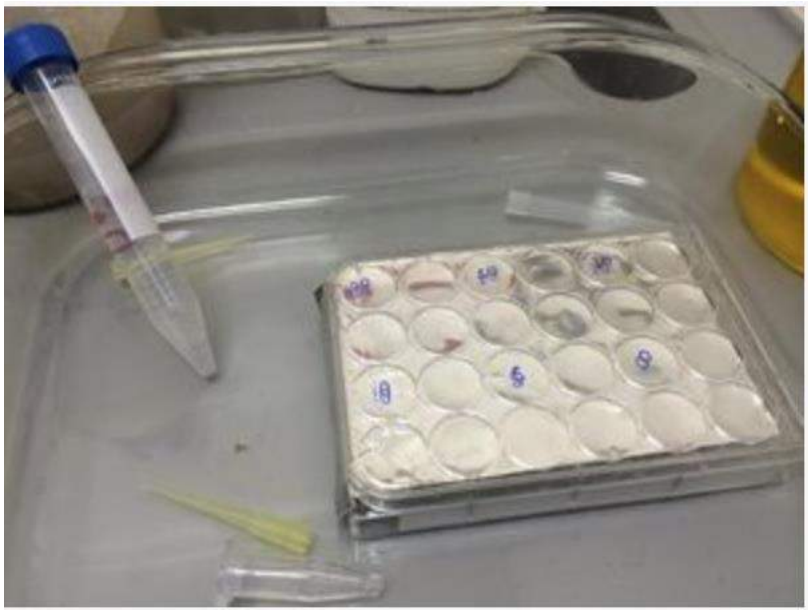
- testing the resistance of the plastic ware and the tolerance of E. coli, Pseudomonas fluorescens and Pseudomonas putida to DCM.

**Notebook:**

First, we tested the resilience of the plastic ware, specifically the 24 well plates, to find out what concentrations of DCM it could cope with.
The plate was set up with the following concentrations of DCM in each well.

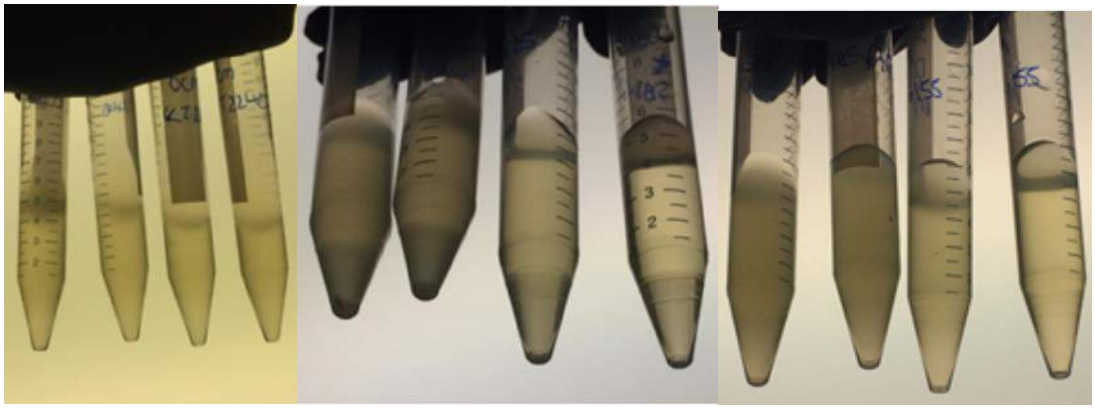
| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | 100mM | | 50mM | | 20mM | |
| B | | | | | | |
| C | 100mM | | 5mM | | 0mM | |
| D | | | | | | |

After leaving the plate overnight covered in foil and the lid to prevent the evaporation of DCM, all wells were intact. This means we can use the plates in our further experiments with DCM.



# Week 1 continued...

To test the tolerance of the bacteria we grew up liquid culture strains using the Growing Liquid Cell Cultures protocol. The strains we used were KT2240 (P. putida), SBW25 (P. fluorescens) and MGG155 (E. coli). Initially we incubated 100$\mu$l of each liquid culture with 5ml LB broth and DCM to make concentrations of 0, 5, 10 and 20mM. Overnight incubations of each strain at an appropriate temperature (30°C for "Pseudomonas" and 37°C for" E. coli") whilst shaking showed that all three strains could tolerate these concentrations, indicating that it's the metabolic intermediates and not the DCM itself that causes the toxicity.
Therefore we repeated the experiment using 0, 20, 50 and 100mM concentrations of DCM. Before doing this we streaked out fresh plates of our cultures using the Agar Plate Preparation and Streaking Plates protocols before growing liquid cultures from these. The results of P. putida, P. fluorescens and E. coli overnight incubation are below:



We ranked the growth in each tube by eye where 0 was no growth and 5 was unhindered growth.

| [DCM]/mM | KT2240 | SBW25 | MGG155 |
|---|---|---|---|
| 0 | 5 | 5 | 5 |
| 20 | 5 | 5 | 5 |
| 50 | 5 | 3 | 4 |
| 100 | 5 | 0 | 2 |

P. putida could tolerate even 100mM, so this strain will be useful when it comes to characterising the dcmA regulatory system. As we now have an idea of what concentrations each strain can tolerate, we can design our experiments for next week where we will obtain growth curves for each strain at various concentrations of DCM.

# Week 2

**Notebook:**
After looking at the qualitative results of the DCM tolerance of various bacterial strains, we decided that a quantitative experiment should follow. We designed an experiment to quantitatively assess the tolerance of bacterial strains (KT2240 (P. putida), SBW25 (P. fluorescens) and MGG155 (E. coli)) to DCM. The plate designs are shown below (where all concentrations are in mM). In the qualitative experiments, P. putida did not show any change in growth up to 100 mM (results shown in figure 1), we wanted to find the upper limit of [DCM] that it could stand (which could act as a positive control as well).

SBW25 (*P. fluorescens*)

| | 1 | 2 | 3 | 4 | 5 | 6 | |
|---|---|---|---|---|---|---|---|
| A | 0 | 20 | 40 | 50 | 60 | 100 | |
| B | 0 | 20 | 40 | 50 | 60 | 100 | |
| C | 0 | 20 | 40 | 50 | 60 | 100 | |
| D | 0 | 20 | 40 | 50 | 60 | 100 | No cells added |

MGG155 (*E. coli*)

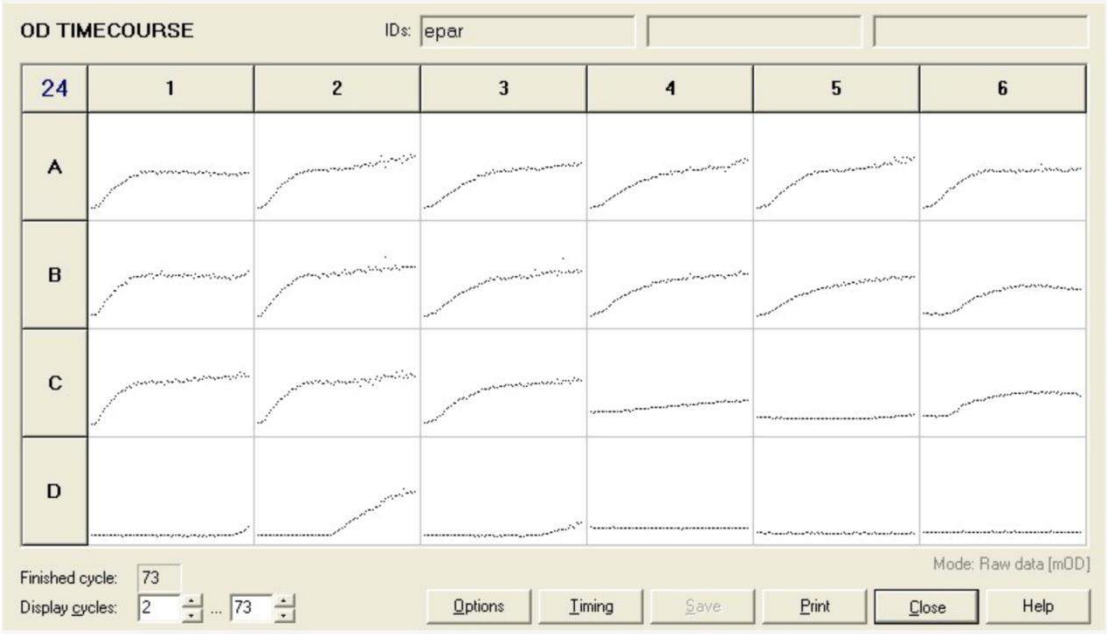
| | 1 | 2 | 3 | 4 | 5 | 6 | |
|---|---|---|---|---|---|---|---|
| A | 0 | 20 | 50 | 80 | 100 | 150 | |
| B | 0 | 20 | 50 | 80 | 100 | 150 | |
| C | 0 | 20 | 50 | 80 | 100 | 150 | |
| D | 0 | 20 | 50 | 80 | 100 | 150 | no cells added |

KT2240 (*P. putida*)

| | 1 | 2 | 3 | 4 | 5 | 6 | |
|---|---|---|---|---|---|---|---|
| A | 0 | 20 | 50 | 100 | 150 | 200 | |
| B | 0 | 20 | 50 | 100 | 150 | 200 | |
| C | 0 | 20 | 50 | 100 | 150 | 200 | |
| D | 0 | 20 | 50 | 100 | 150 | 200 | No cells added |

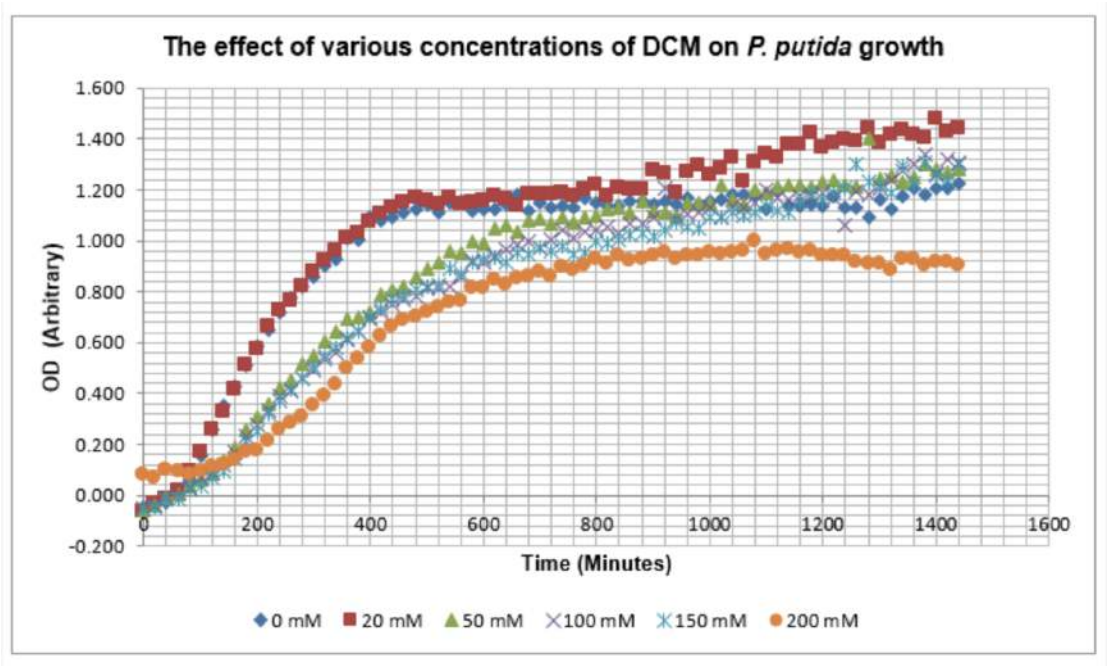
# Week 2 continued...

To measure the changing absorbance in these plates we used the Measuring Cell Density Over Time protocol. By the end of this week we had results for the Putida plate, which are shown below.

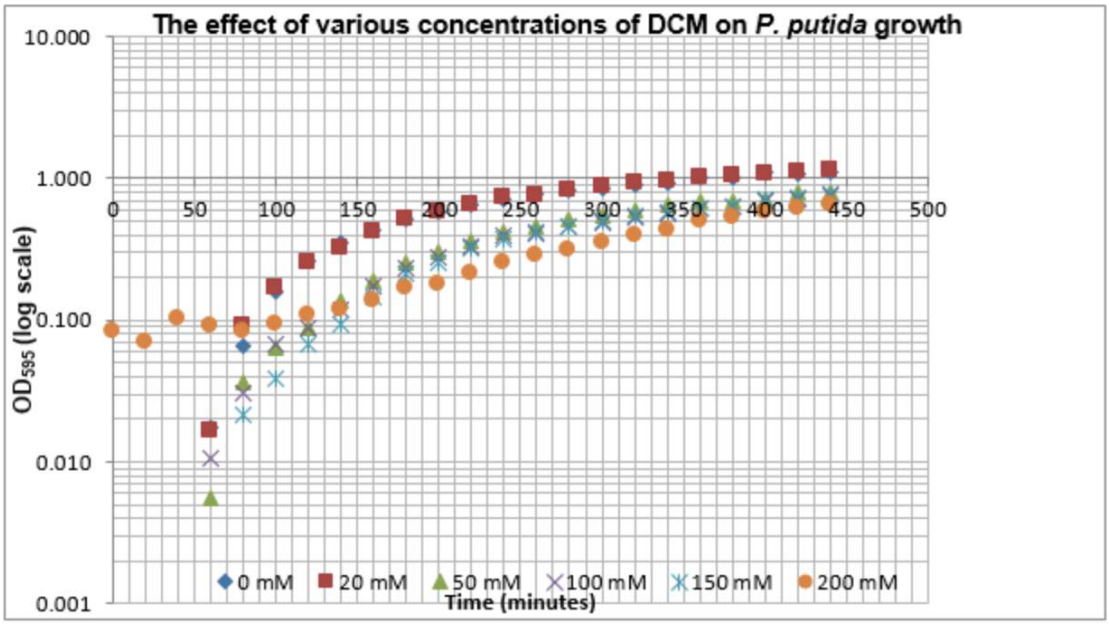



As it can be seen from the results, the control wells D1, D2 and D3 all show signs of contamination (perhaps a very small quantity were accidentally transferred during pipetting) since these wells only contain DCM and so should not change over time. Another possible source of error for these results is a white powder we found on the top of the plate when removing it. This could have scattered light and so affected the OD reading. These wells as a result were discarded during data analysis. It appears that wells C4 and C5 are outliers as well and this may due to fewer cells being transferred, since we did not mix the test tube thoroughly before transferring the bacterium to the plates and also due to the small volume in the test tube we had to tilt it to transfer the bacteria, this means since these were the last wells to be pipetted, this is a likely cause for these outliers. From these results, the following graph was plotted (all values are normalised to the control wells in row D):

# Week 2 continued...



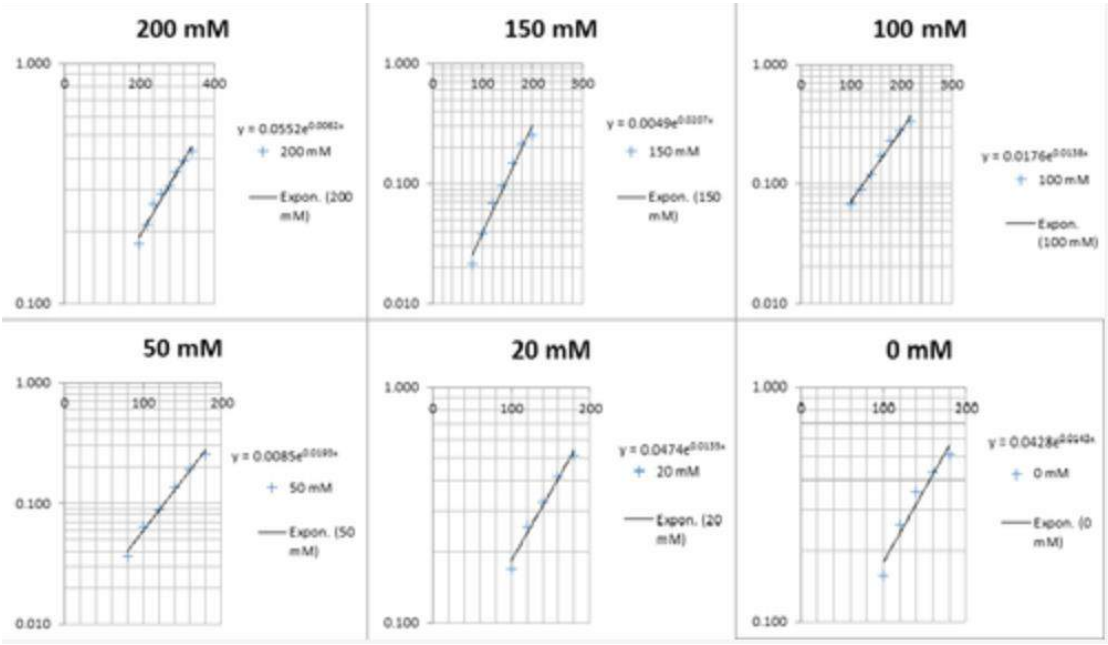

The data on P. putida above was replotted so that it was a semilog graph:




The linear part of these graphs needed to be taken as this represents the log phase of growth, which is the phase we are measuring. The time points in which each curve was linear was assessed by eye and the following graphs were obtained. The following equation was then used for points that were on the line of best fit of these graphs.

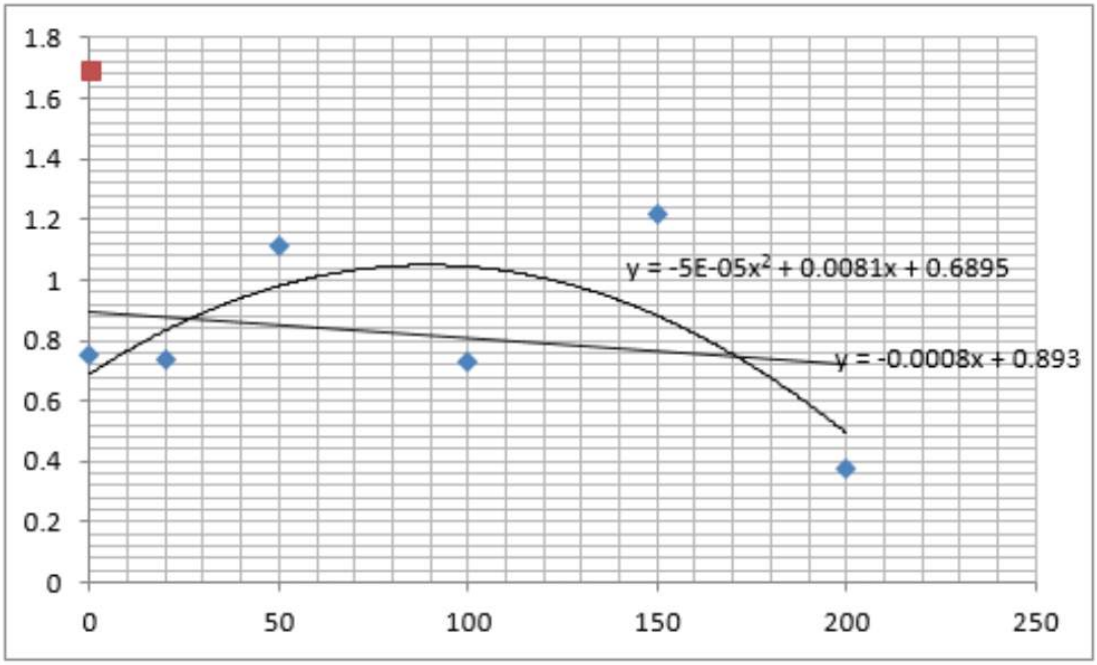
# Week 2 continued...

$$\mu = \frac{lnOD_2 - lnOD_1}{t_2 - t_1} = \frac{2.303(logOD_2 - logOD_1)}{t_2 - t_1}$$



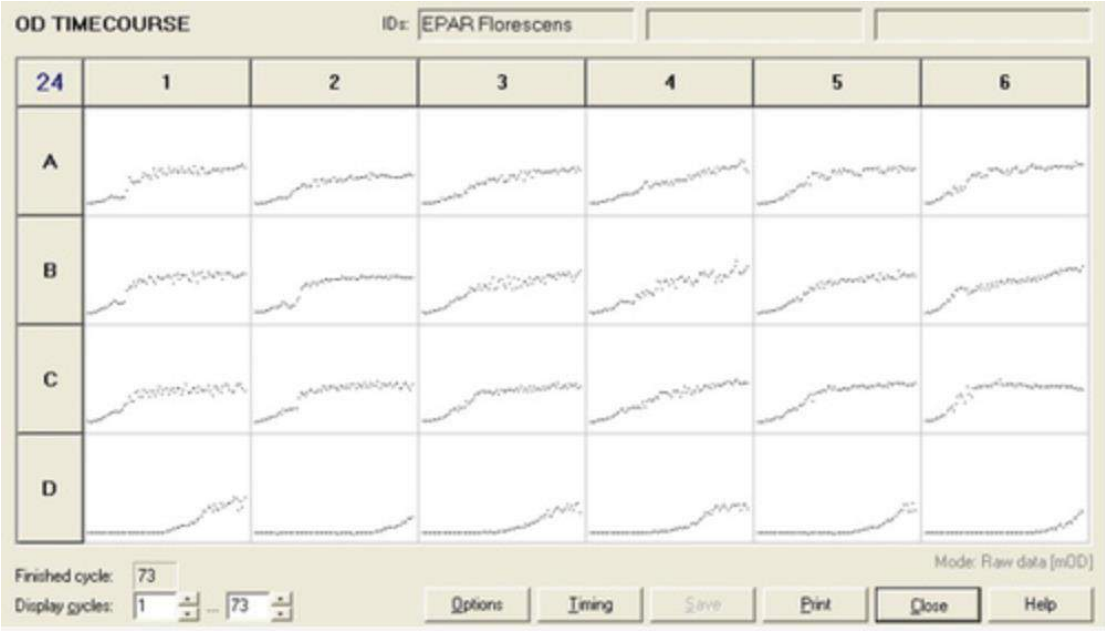

| [DCM] (mM) | Equation | Result ($\mu$) (min$^{-1}$) | Result ($\mu$) (hr$^{-1}$) |
|---|---|---|---|
| 0 | $\mu = \frac{ln0.43 - ln0.26}{160 - 120}$ | 0.0126 | 0.755 |
| 20 | $\mu = \frac{ln0.415 - ln0.325}{160 - 140}$ | 0.0122 | 0.733 |
| 50 | $\mu = \frac{ln0.191 - ln0.063}{160 - 100}$ | 0.0185 | 1.109 |
| 100 | $\mu = \frac{ln0.278 - ln0.171}{200 - 160}$ | 0.0121 | 0.729 |
| 150 | $\mu = \frac{ln0.212 - ln0.094}{180 - 140}$ | 0.0203 | 1.220 |
| 200 | $\mu = \frac{ln0.351 - ln0.213}{300 - 220}$ | 0.00624 | 0.375 |

# Week 2 continued...

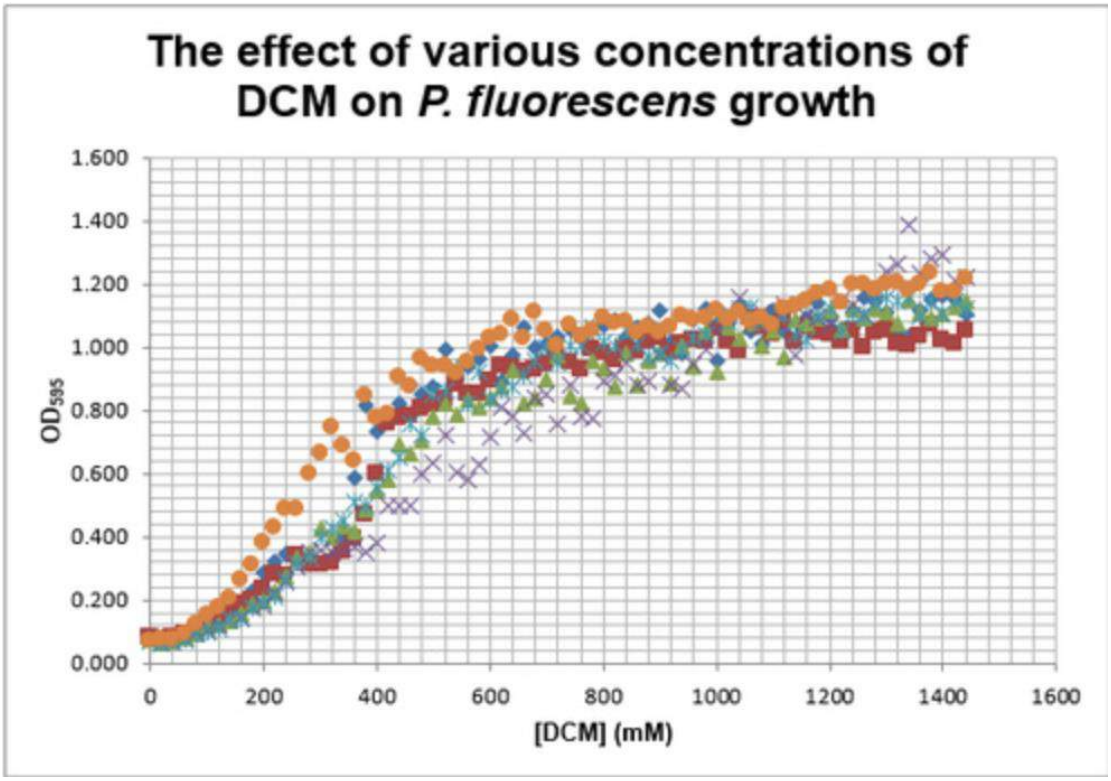


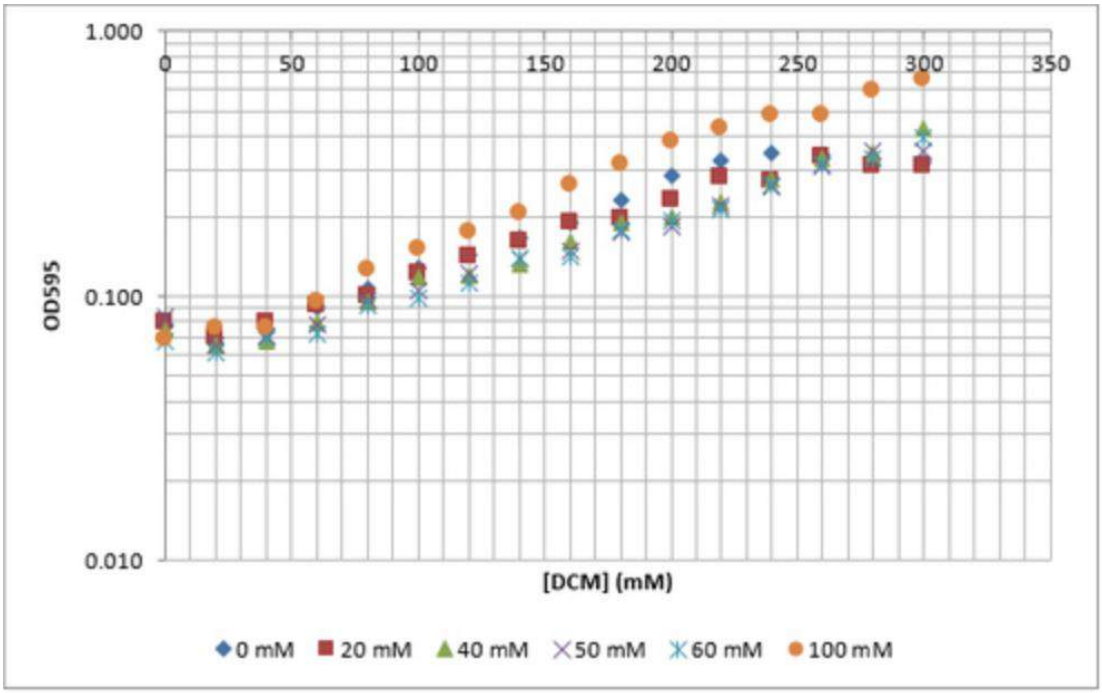

The graph gave two conclusions, depending on the type of line that was fitted to the data, we will repeat these measurements and determine the statistical significance in other to assign the correct one.

This experiment was repeated using P. fluorescens and the results are as follows:



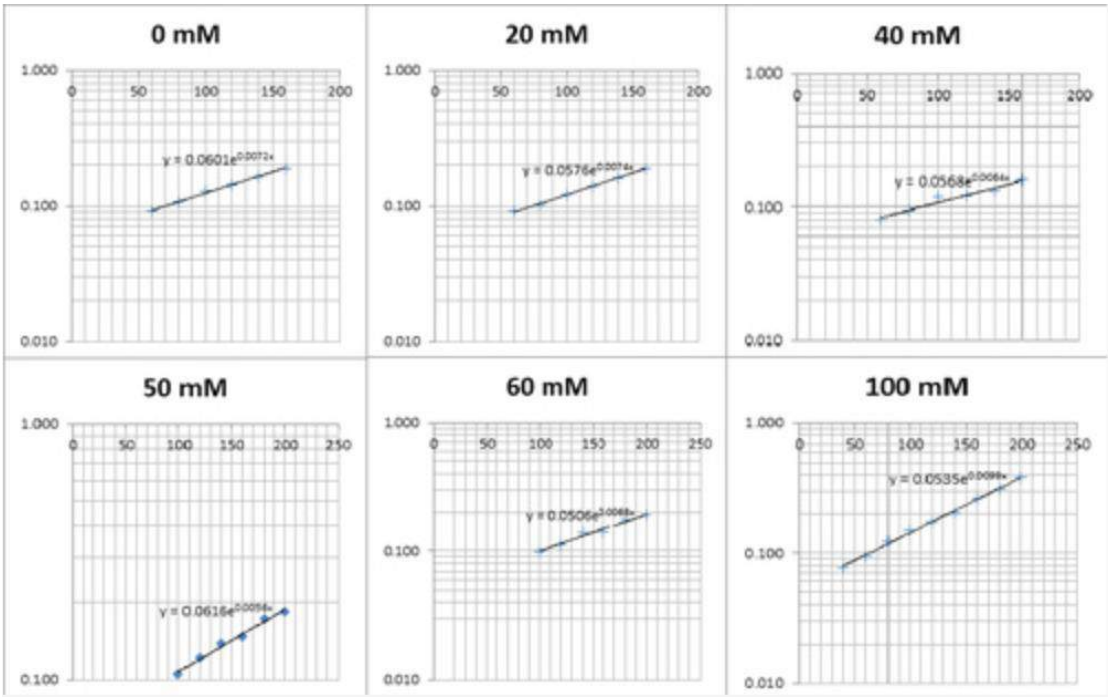
# Week 2 continued...



# Week 2 continued...

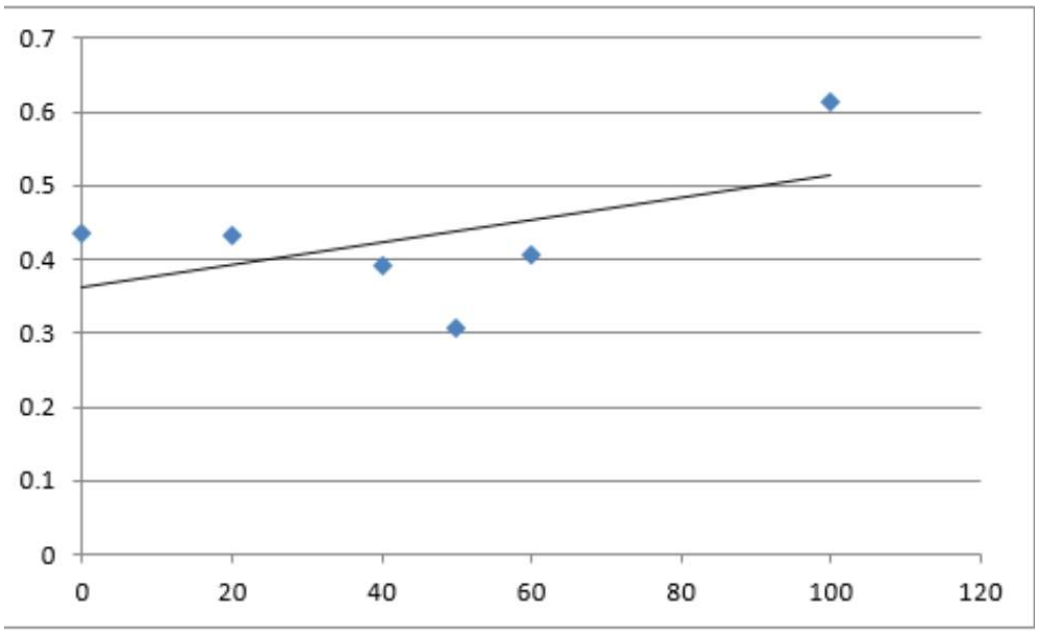
Straight lines from Graph C were then taken to calculate μ values as previously described. The results are shown below:



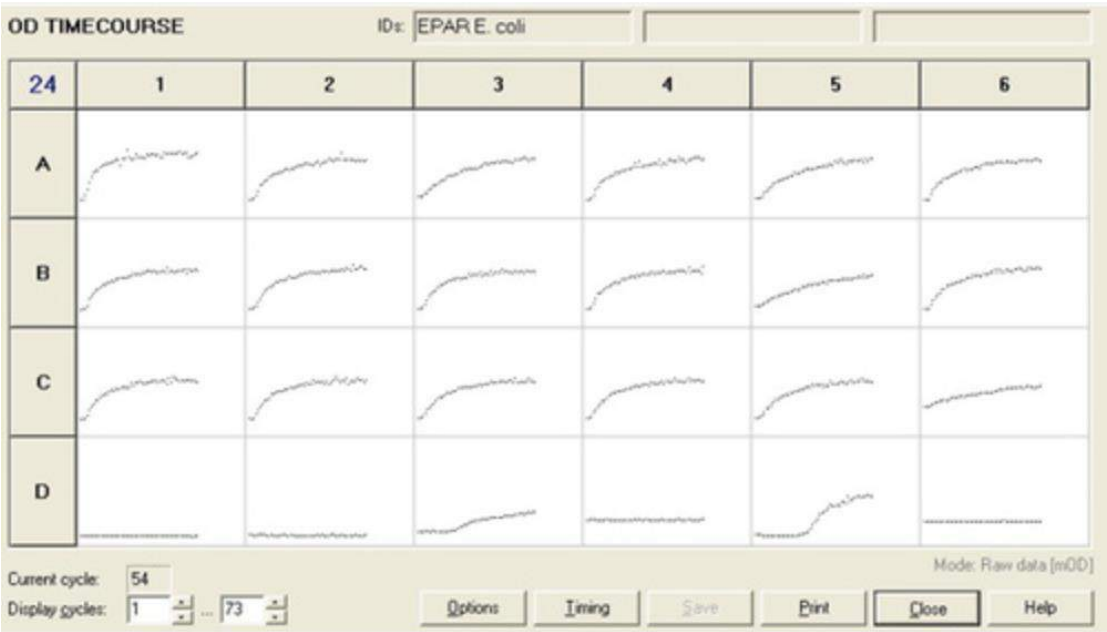

| [DCM] (mM) | Equation | Result ($\mu$) ($min^{-1}$) | Result ($\mu$) ($hr^{-1}$) |
|---|---|---|---|
| 0 | $\mu = \frac{ln0.143 - ln0.107}{120 - 80}$ | 0.00725 | 0.435 |
| 20 | $\mu = \frac{ln0.188 - ln0.122}{160 - 100}$ | 0.00721 | 0.432 |
| 40 | $\mu = \frac{ln0.160 - ln0.095}{160 - 80}$ | 0.00652 | 0.391 |
| 50 | $\mu = \frac{ln0.184 - ln0.122}{200 - 120}$ | 0.00514 | 0.308 |
| 60 | $\mu = \frac{ln0.193 - ln0.098}{200 - 100}$ | 0.00678 | 0.407 |
| 100 | $\mu = \frac{ln0.262 - ln0.174}{160 - 120}$ | 0.0102 | 0.614 |

# Week 2 continued...

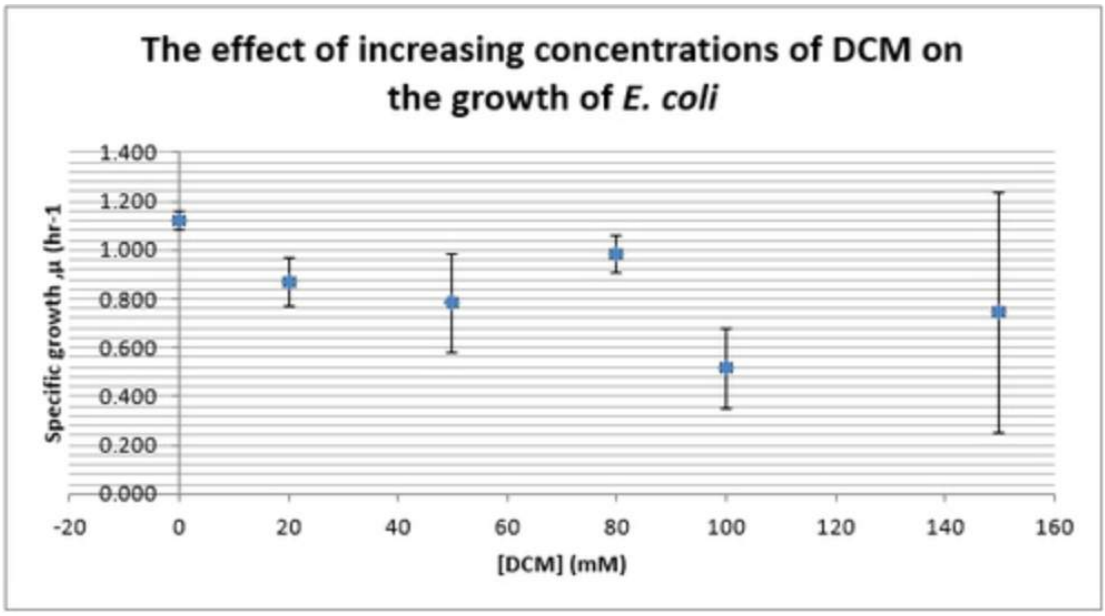
The graph below plots the data in the table for μ.




Again, two lines can be drawn, giving contrasting conclusions, we will perform repeats and determine the statistical significance of these data points. The experiments were then repeated with E. coli and the results are shown below.



# Week 2 continued...




To test significance we will perform an ANOVA test on the data. It appears prima facie that the change is not significant, this is probably due to contamination and/or evaporation of the DCM from the wells.

In order to pinpoint the source of the contaminated control wells, I set up an experiment with Jack where we measured the UV-vis spectrum (initially between 800 nm and 200 nm) of 200 mM DCM (in media) with the baseline corrected to the media. We could not use pure DCM as it melted the cuvettes. The only peak from this broad scan was approximately located at 335 nm. This scan was performed at approximately 3:35. This peak was off of the absorbance chart, however we predict that if DCM can penetrate the plate seal, it will do so on the 24 hour time scale and will evaporate almost entirely and the peak will disappear. If this indeed happens after 24 hours then we can infer that the plate seals unsticking are the source of the contamination. There will be two conditions that we have not accurately repeated in this experiment compared to the plate reader experiments. These are the temperature (as the cuvette was left at room temperature rather than 30℃ and 37℃). The other control is the type of plastic of the plate reader, this may affect adhesion of the glue on the plate seal. There are also no cells in this, the interaction of DCM with the cells may produce something that might affect the seal of the plate.

# Week 3

**Notebook:**
ANOVA tests were performed on all three of the data sets and this revealed that there is no statistical significance between the groups.

***P. fluorescens***

| 0 | 20 | 40 | 50 | 60 | 100 |
|---|---|---|---|---|---|
| 0.327183 | 0.402214 | 0.293848 | 0.085851 | 0.368734 | 0.588498 |
| 0.391008 | 0.452191 | 0.483549 | 0.447762 | 0.422078 | 0.713009 |
| 0.604234 | 0.44056 | 0.455118 | 0.326693 | 0.563137 | 0.53723 |

Anova: Single Factor

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| 0 | 3 | 1.322425 | 0.440808 | 0.021049 |
| 20 | 3 | 1.294964 | 0.431655 | 0.000684 |
| 40 | 3 | 1.232515 | 0.410838 | 0.010467 |
| 50 | 3 | 0.860306 | 0.286769 | 0.03394 |
| 60 | 3 | 1.35395 | 0.451317 | 0.010089 |
| 100 | 3 | 1.838737 | 0.612912 | 0.008172 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 0.163266 | 5 | 0.032653 | 2.321262 | 0.107737 | 3.105875 |
| Within Groups | 0.168804 | 12 | 0.014067 | | | |

# Week 3 continued...

***P. putida***

| 0 | 20 | 50 | 100 | 150 | 200 |
|---|---|---|---|---|---|
| 0.017 | 0.019 | 0.018 | 0.015 | 0.024 | 0.002 |
| 0.005 | 0.011 | 0.019 | 0.015 | 0.016 | 0.015 |
| 0.012 | 0.010 | 0.014 | | | 0.006 |

Anova: Single Factor

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| 0 | 3 | 0.033602 | 0.011201 | 3.89E-05 |
| 20 | 3 | 0.040094 | 0.013365 | 2.29E-05 |
| 50 | 3 | 0.050483 | 0.016828 | 4.71E-06 |
| 100 | 2 | 0.029539 | 0.014769 | 9.1E-08 |
| 150 | 2 | 0.040046 | 0.020023 | 3.98E-05 |
| 200 | 3 | 0.023182 | 0.007727 | 4.22E-05 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 0.000237 | 5 | 4.75E-05 | 1.844934 | 0.191651 | 3.325835 |
| Within Groups | 0.000257 | 10 | 2.57E-05 | | | |
| Total | 0.000495 | 15 | | | | |

# Week 3 continued...

***E. coli***

| 0 | 20 | 50 | 80 | 100 | 150 |
|---|---|---|---|---|---|
| 1.09763 | 0.935894 | 0.561079969 | 1.062168 | 0.627193 | 1.010874 |
| 1.162311 | 0.753883 | 0.955130754 | 0.916651 | 0.329578 | 1.045354 |
| 1.097515 | 0.912182 | 0.836483206 | 0.966437 | 0.587468 | 0.177355 |

Anova: Single Factor

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| 0 | 3 | 3.357455632 | 1.119152 | 0.001397 |
| 20 | 3 | 2.601959016 | 0.86732 | 0.009792 |
| 50 | 3 | 2.352693929 | 0.784231 | 0.040867 |
| 80 | 3 | 2.945255683 | 0.981752 | 0.00547 |
| 100 | 3 | 1.544239211 | 0.514746 | 0.02611 |
| 150 | 3 | 2.233582144 | 0.744527 | 0.241561 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 0.649943 | 5 | 0.129989 | 2.398344 | 0.09956 | 3.105875 |
| Within Groups | 0.650391 | 12 | 0.054199 | | | |
| Total | 1.300334 | 17 | | | | |

To allow for possible contamination we will repeat these experiment using air-tight glass conical flask to prevent spillage between wells, DCM reacting with the plastic or evaporation of the DCM. This week we have also finally managed to make media for the DM4 to grow in. Up until now all our media had been falling out of solution but we tried two different recipes with the ingredients sent to us from the media kitchen. The first recipe was as follows:

- 0.83 mL $MgSO_4$
- 100 uL trace elements
- 99.17 mL minimal media

# Week 3 continued...

Where minimal medium used contained:
2 g/L KH2PO4
4 g/L Na2HPO4.2H2O
1 g/L (NH4)2SO4
1 g/L MgSO4.7H2O

To this 1ml of trace elements solution was added which contained:
1 g/L FeSO4.7H2O
1 g/L MnSO4¬H2O
0.25 g/L (NH4¬)6Mo7O24.4H2O
1 g/L H3BO3
0.25 g/L CuCl2.2H2O
0.25 g/L ZnCl2
0.1 g/L NH4VO3
0.25 g/L Co(NO3)2.6 H2O
25 g/L Ca(NO3)2

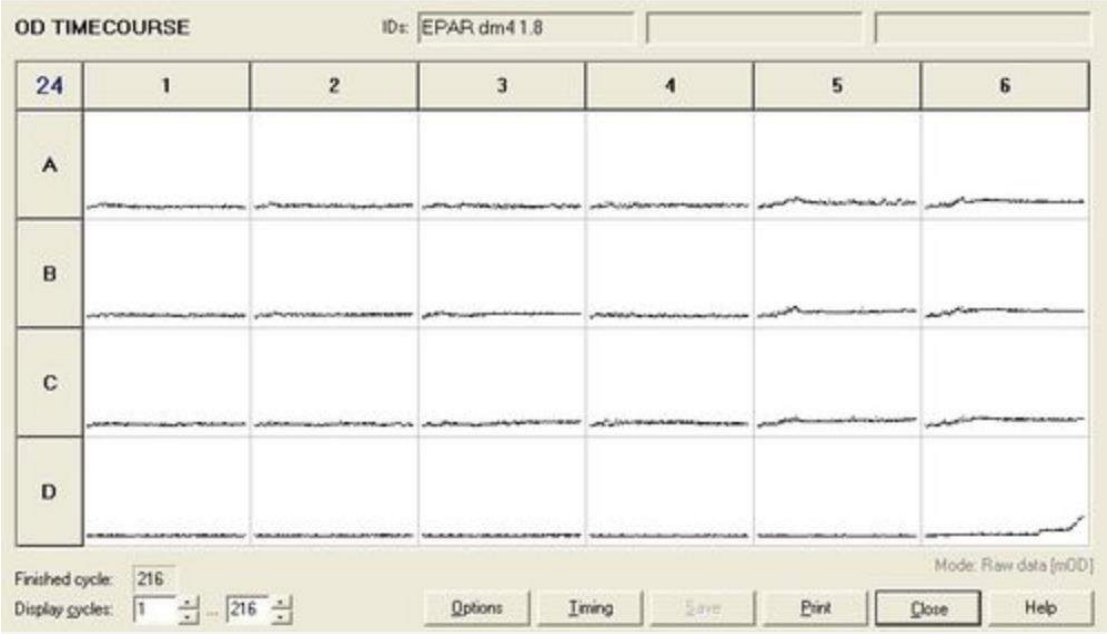
The second recipe was as follows, although we only made 100ml of solution and therefore divided all the amounts added by 10.

| MP Media Recipe | | | FOR STOCK SOLUTIONS: | | FOR 1 L MEDIA: | |
|---|---|---|---|---|---|---|
| | | Molecular Weight (g) | CONCENTRATION | ADD TO 1 L $dH_2O$ | FINAL CONCENTRATION | ADD |
| **PIPES** | $C_8H_8N_2O_6S_2$ | 302.37 | 300 mM (10X) | 90.711 g | 30 mM | 100 mL |
| **P solution** | | | (100X) | | | |
| | $K_2HPO_4$ •3 $H_2O$ | 228.22 | 145.0 mM | 33.1 g | 1.45 mM | 10 mL |
| | $NaH_2PO_4$ •$H_2O$ | 137.99 | 187.69 mM | 25.9 g | 1.88 mM | |
| **$MgCl_2$** | $MgCl_2$ •6 H2O | 203.3 | 2 M (4000X) | 406.6 g | 0.5 mM | 250 µL |
| **$(NH4)_2SO_4$** | $(NH4)_2SO_4$ | 132.14 | 2 M (250X) | 264.28 g | 8 mM | 4 mL |
| **$CaCl_2$** | $CaCl_2$ •2 $H_2O$ | 147.02 | 2 M (100,000X) | 294.04 g | 20 µM | 10 µL |
| **C7-Metals** | sodium citrate | | (1000X) | ADD TO 500 mL $dH_2O$ | | |
| MIX IN ORDER LISTED ↓ | ($Na_3C_6H_5O_7$ •2 $H_2O$) | 294.1 | 45.53 mM | 6705.5 mg | 45.6 µM | 1 mL |
| | $ZnSO_4$ •7 $H_2O$ | 287.54 | 1.2 mM | 172.52 mg | 1.2 µM | |
| | $MnCl_2$ •4 $H_2O$ | 197.9 | 1.0 mM | 99 mg | 1 µM | |
| | $FeSO_4$ •7 $H_2O$ | 278.01 | 18 mM | 2502 mg | 18 µM | |
| | $(NH_4)_6Mo_7O_{24}$ •4 $H_2O$ | 1235.86 | 2 mM | 1235.6 mg | 2 µM | |
| | $CuSO_4$ •5 $H_2O$ | 249.68 | 1 mM | 124.8 mg | 1 µM | |
| | $CoCl_2$ •6 $H_2O$ | 237.93 | 2 mM | 237.9 mg | 2 µM | |
| | $Na_2WO_4$ •2 $H_2O$ | 329.85 | 0.33 mM | 54.4 mg | 0.33 µM | |
| **milliQ-$H_2O$** | | | | | | 885 mL |

We decided to grow DM4 in 5 mL Duran flasks at 30℃, these flasks are airtight to prevent the DCM from evaporating. From our research in the literature we decided to use 120 mM methanol as the carbon source. The DM4 that we grew up in the different types of media appeared to grow. We decided to make a frozen stock of each of the 4 sub cultures. We also made a 24-well plate containing DM4 bacteria with various concentrations of methanol and DCM.

# Week 4

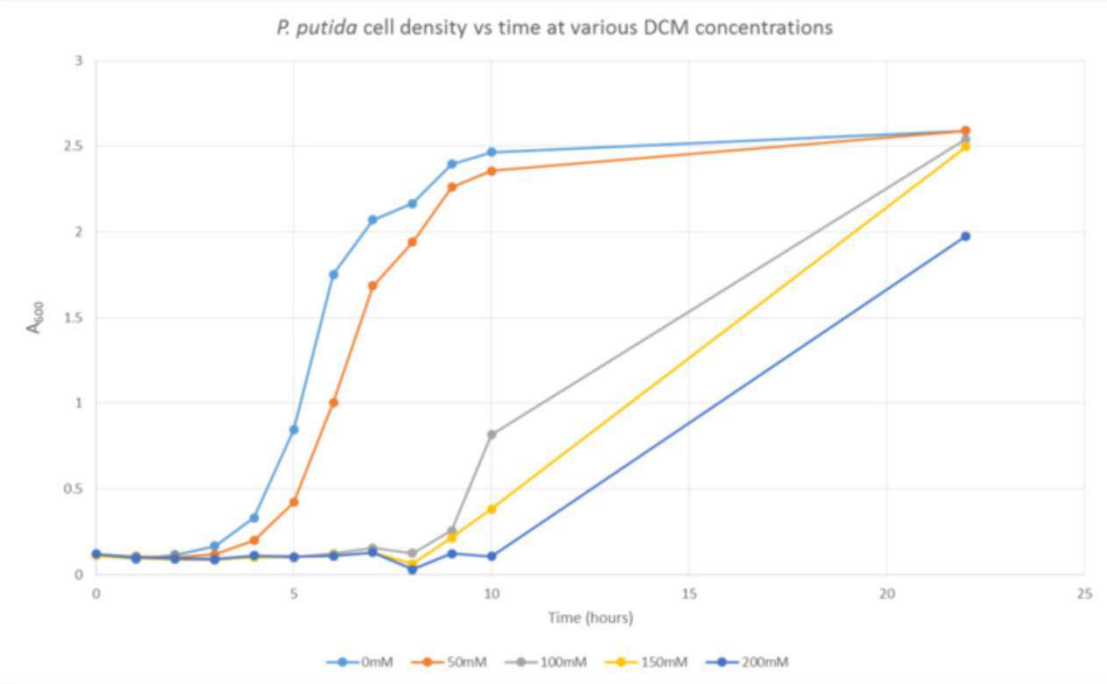
**Notebook:**
Unfortunately, none of the DM4 grew in the 24 well plate. The results of the plate are shown below.




In order to assess why this has happened we grew up DM4 from frozen stock in the air-tight glass conical flasks at 20mM DCM and 120mM methanol. This didn't work because although we got growth, the bacteria weren't pink but yellow indicating there had been contamination. I have therefore made liquid cultures from all 4 of the frozen stocks we have of DM4 in 120mM methanol and 10ml growth medium.

We are also growing Putida and E.coli in the same glass air-tight conical flasks in order to find out how tolerant they are and get rid of the aforementioned sources of contamination. We first grew Putida at concentrations of 0, 50, 100, 150 and 200mM DCM in duplicate and these were the results:

# Week 4 continued...




The presence of the DCM either significantly slows the growth rate of the cells, or prevents them from growing and is evaporating over time. In order to test this I grew Putida in 0 and 50mM DCM and took a sample after 6 hours. By comparison with the initial growth curve, it is possible to distinguish between the two possibilities.

# Week 4 continued...

This week our aim is to get started on assembling and sequencing biobrick plasmids. At the moment there are 8 constructs we are aiming to make and submit and they are as follows:

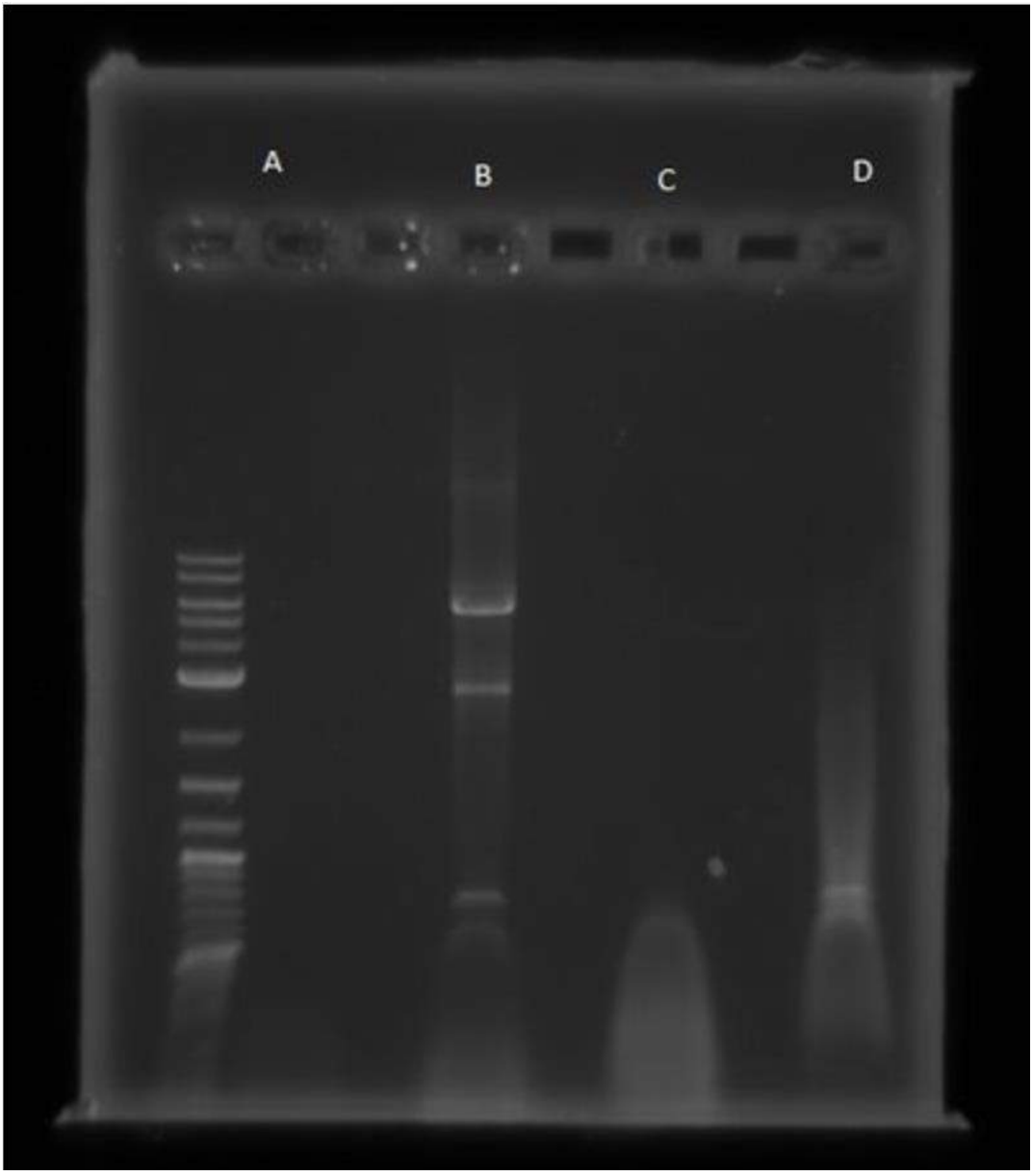
| Reaction Name | Template Files used | Construct |
|---|---|---|
| A | Ecoli-dcmA-noMicro-RE | pSB1C3-RBS-dcmA-Ecoli |
| | Psb1c3 | |
| B | Putida-dcmA-noMicro-RE | pSB1C3-RBS-dcmA-pseud |
| | Psb1c3 | |
| C | Ecoli-dcmA-Micro-RE | Psb1c3-RBS-microtag-dcmA-Ecoli |
| | Psb1c3 | |
| D | Putida-dcmA-Micro-RE | pSB1C3-RBS-microtag-dcmA-Pseud |
| | Psb1c3 | |
| E | PdcmA-sfGFP | pSB1C3-PdcmA-sfGFP |
| | Psb1c3 | |
| F | Construct 2.2 | pSB1C3-Pamp-tetR-term-Ptet-RBS-dcmR |
| | psb1c3 | |
| G | Putida-dcmA-micro-formaldehyde | pSB1C3-RBS-microtag-formaldehydeDH |
| | Psb1c3 | |
| H | Putida-micro-GFP | Psb1c3-RBS-microtag-GFP |
| | Psb1c3 | |

# Week 4 continued...

I have begun work to construct parts E and F by PCRing the fragments and backbones with the relevant primers. The PCR reactions were as follows:

| Pamp-tetR-term-Ptet-RBS-dcmR | Reaction | Fragment Length |
|---|---|---|
| Pamp-tetR-term-Ptet-RBS-dcmR | A | 1800 |
| pSB1C3 for Pamp-tetR-term-Ptet-RBS-dcmR | B | 2100 |
| PdcmA-sfGFP | C | 1600 |
| pSB1C3 for PdcmA-sfGFP | D | 2100 |

We then ran the PCR products on the gel and these were the results:



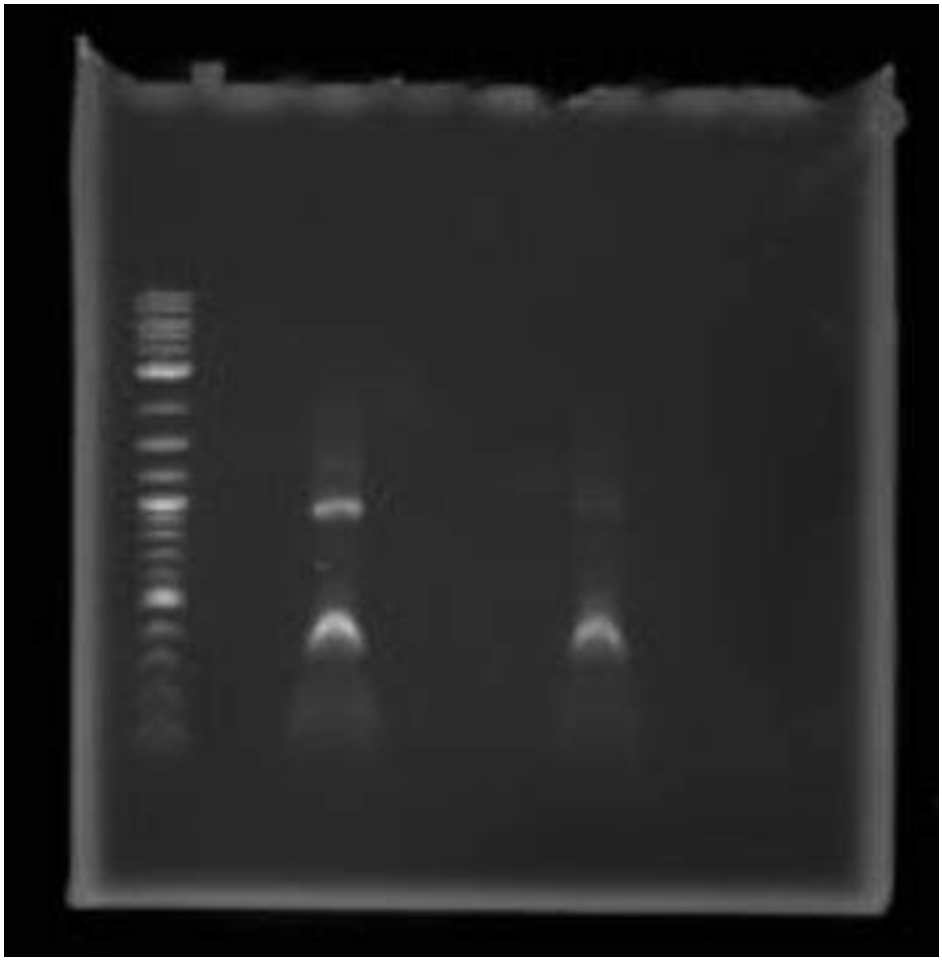
# Week 4 continued...

For reaction A, there is no DNA band showing our PCR didn't work. This PCR was to join 3 G blacks with complementary ends together which team B is trying to do with a Gibson assembly so hopefully they'll have more success. Reaction B has shown bands which would seem to be to big for the fragment we wanted, whereas in reaction D the DNA appears too small. In lane C there also seems to be nothing.

Also, we hope to improve on the microcompartment part in the registry, and so this week we have sequenced this part ourselves. Using the Nanodrop protocol, I found the concentration of the DNA frozen stock of the pSB1C3 microcompartment, diluted it appropriately and sent it for sequencing. This sequencing reaction only covered up to the first 800 bases of the sequence, and so I have designed 4 more primers which should ensure the entire part is sequenced and sent it for sequencing. The sequence of the part itself matches that on the registry, however there seems to be part of the biobrick prefix missing. In order to check this we will carry out restriction digests using EcoR1, Spe1, Pst1 and Xba1 and check the fragment lengths.

# Week 5

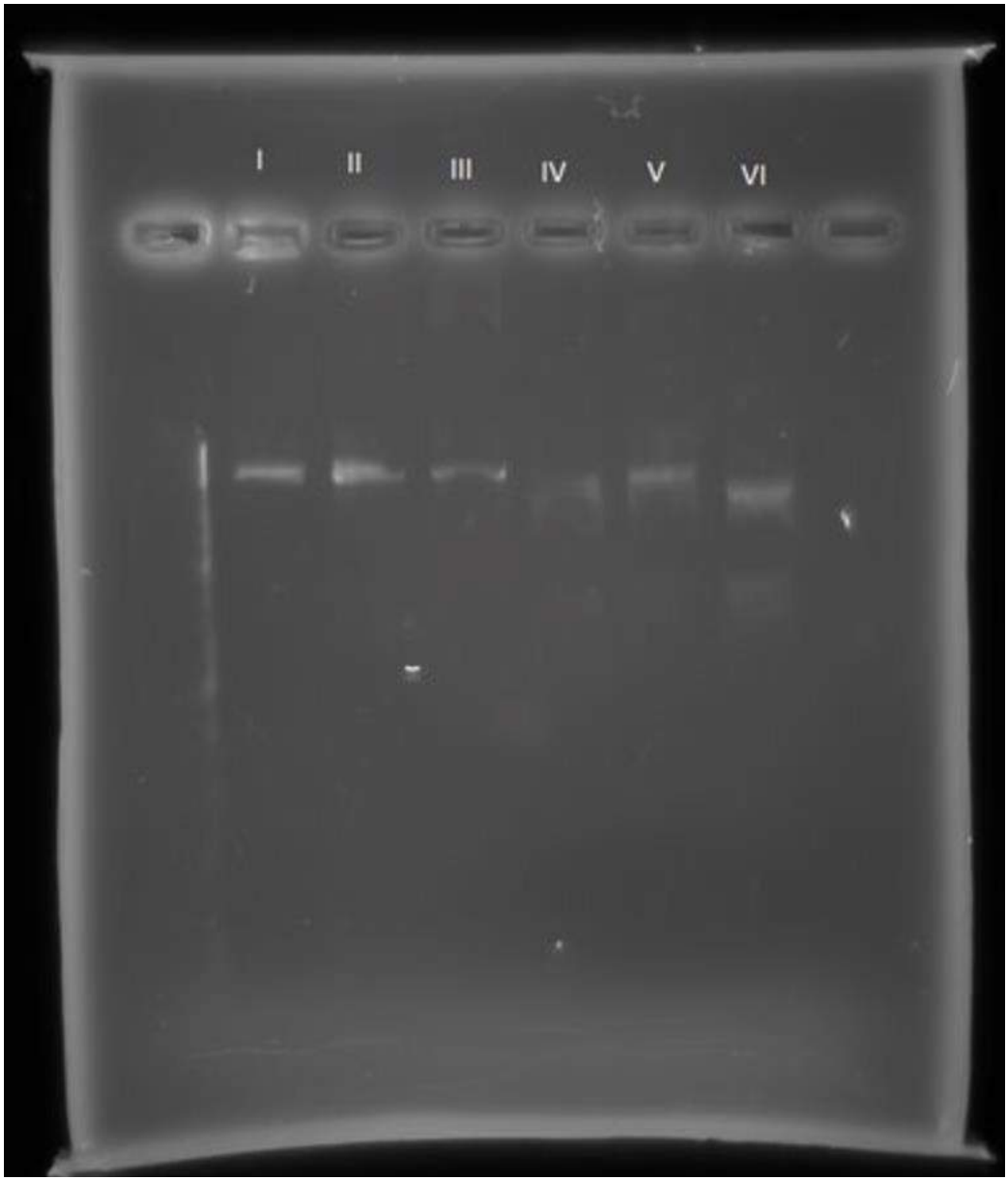
**Notebook:**
For the PCR reactions last week when trying to amplify the biobrick backbone, I have realised that the site where my primers were meant to bind are actually the bits missing from the pSB1C3 microcompartment backbone, and this is probably why the backbone wasn't amplified. Therefore I will attempt to do these reactions again using a different template - the pSB1C3 containing the gene for mCherry.
I first ran a PCR with the sfGFP fragment we had as a G block at 72 degrees C and with an elongation time of 2 minutes and 10 seconds. I used a 1 in 10 dilution of the G block and used 1ul and 5ul of this in two separate PCR reactions. I then ran the PCR product on a 1% agarose gel:



The two bands we got at roughly 1100bp and 400bp were both smaller than the 1600bp we were expecting.

# Week 5 continued...

Also these are the results of the restriction enzyme digest that I set up with all 4 biobrick restriction enzymes:




The reactions were as follows:
I. pSB1C3-microcompartments with EcoR1 and Spe1
II. pSB1C3-microcompartments with Xba1 and Pst1
III. pSB1C3-microcompartments with Xba1 and Spe1
IV. pSB1C3-mCherry with EcoR1 and Spe1
V. pSB1C3-mCherry with Xba1 and Pst1
VI. pSB1C3-mCherry with Xba1 and Spe1

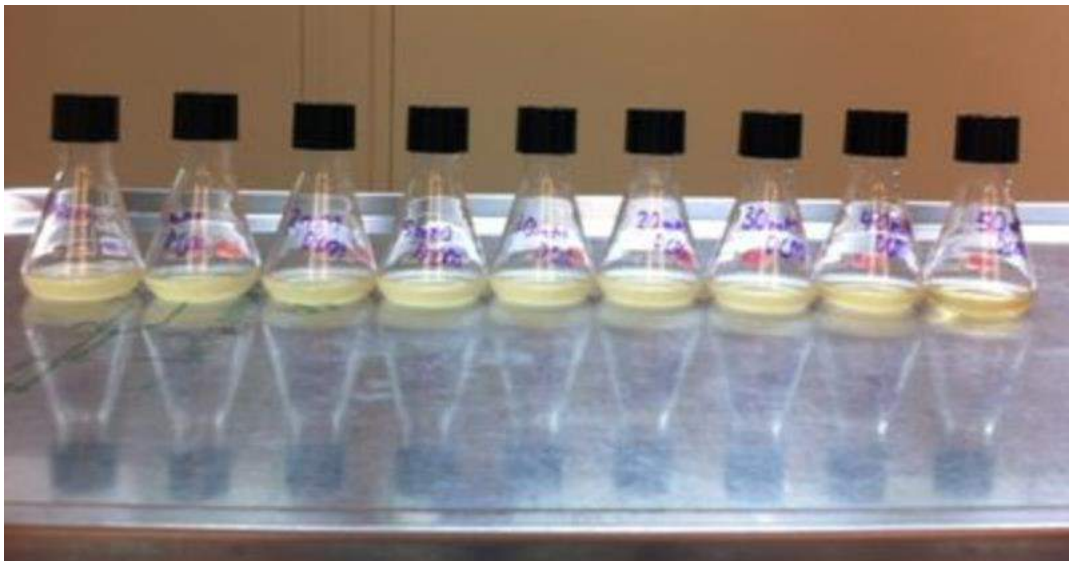
# Week 5 continued...

With the pSB1C3-microcompartments plasmid there is only one band, indicating it has only been cut once with one restriction enzyme each time. With the pSB1C3-mCherry there are two bands, indicating the plasmid has been cut twice. To further find out which sites are present on the pSB1C3-microcompartments plasmid I will send it for further sequencing, to cover both the biobrick prefix and suffix. This sequencing has confirmed what our RE digest showed us:

| | |
|---|---|
| Spe1 | Not present |
| Pst1 | Present |
| EcoR1 | Present |
| Xba1 | Not present |

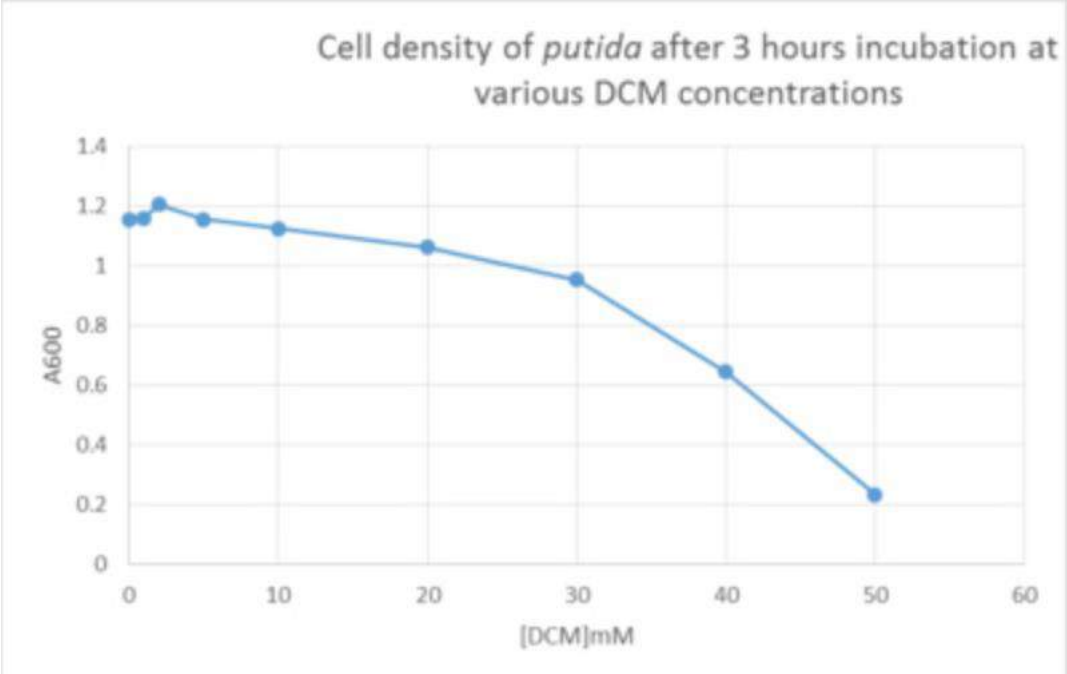
So I will now design primers to amplify the microcompartment genes and add the correct biobrick suffix/prefix at either end.

The results of leaving putida to grow for 6 hours without opening the flasks gave readings of A600 of 1.675 at 0mM DCM and 0.023 at 50mM DCM. This means that it is likely the only reason the bacteria are growing at such high DCM concentrations is that the DCM is evaporating each time the flask is opened. In order to solve this, I will set up flasks with 0,1,2,5,10,20,30,40 and 50mM DCM with putida and just observe their growth. After observing these flasks for 3 hours, there appeared to be growth in all of them, even at 50mM DCM (although not as much as the others).



# Week 5 continued...

I then took samples out of these flasks and these were the results:



# LAB BOOK

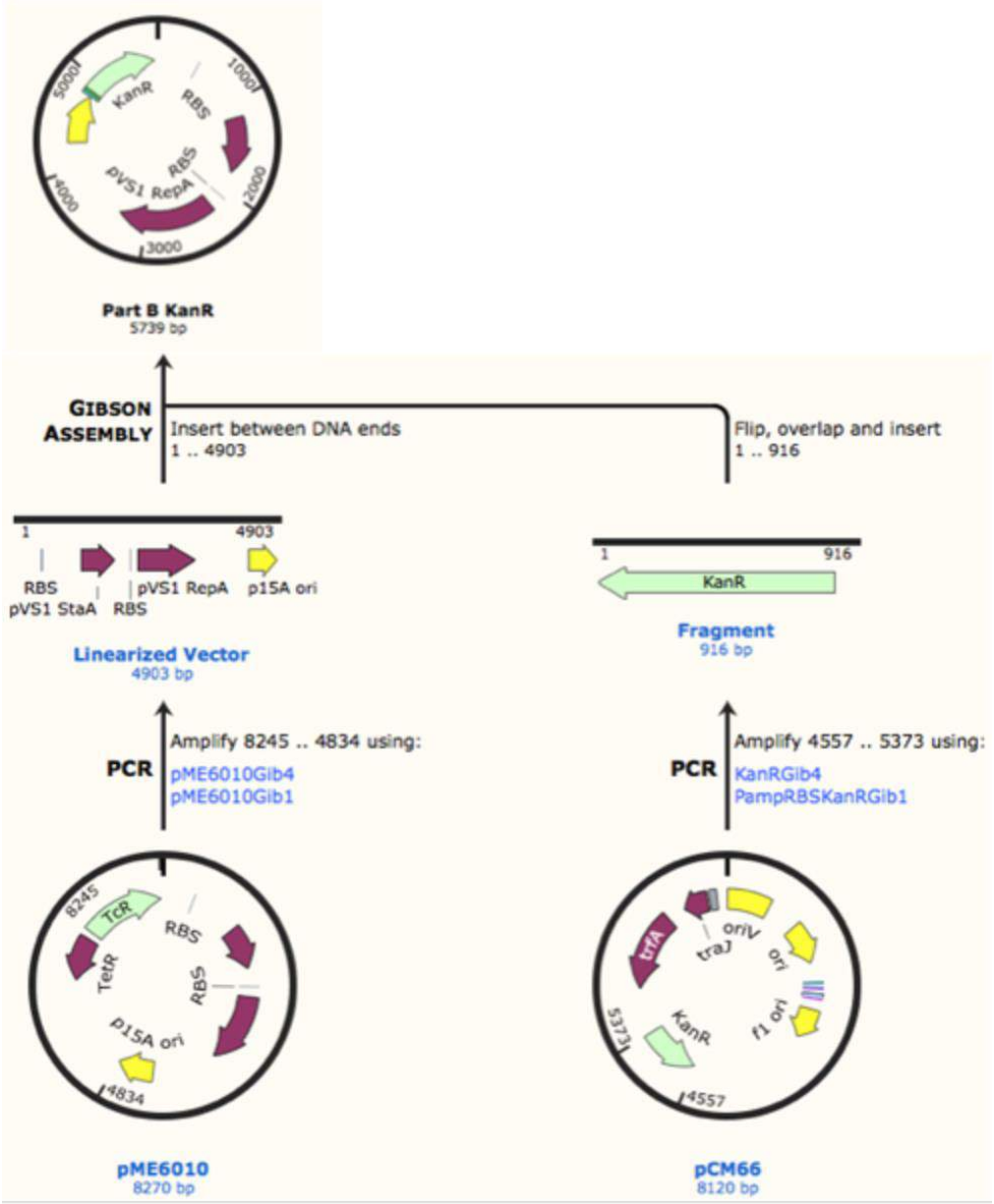
## Biosensor

# Week 1

**Tasks completed:**

- First attempt at swapping resistance gene for plasmid pME6010 from tetracycline to kanamycin

**Notebook**:
First, we designed forward and reverse primers for KanR (with native RSB and promoter) which we isolated from plasmid pCM66. The 5' ends were complementary to the insert region of pME6010 (reaction 1). We then designed forward and reverse primers to amplify the pME6010 backbone (reaction 2). Further to this we redesigned each set of primers to incorporate an ampicillin promoter and optimised RBS (https://salis.psu.edu/software/) (B reactions) instead of the native promoter region (A reactions). This process is described in the following map:



# Week 1 continued...

The designed plasmids were as follows:

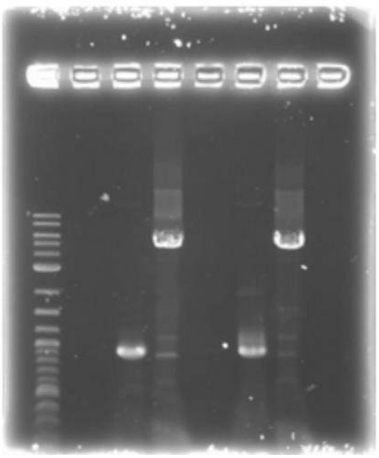
| PCR reaction | Primer name | Primer sequence | Fragment length / bp |
|---|---|---|---|
| 1A | KanRGib3 | ggagcctatggaaaaacggcagataaaaatatatcatcatgaacaataaaactgtctgcttaca | 920 |
| | KanRGib4 | ctatcgtttccacgagcaaattagaaaaactcatcgagcatcaaatgaaactgcaat | |
| 1B | PampRBSKanRGib1 | ggagcctatggaaaaacggcttcaaatatgtatccgctcatgagacaatTTATCTTCCGAGTCCTAGGAGGTATTAATTAatgagccatattcaacggg | 920 |
| | KanRGib4 | ctatcgtttccacgagcaaattagaaaaactcatcgagcatcaaatgaaactgcaat | |
| 2A | pME6010Gib3 | atgatgatatatttttatctgccgtttttccataggctccgcc | 4868 |
| | pME6010Gib4 | tgctcgatgagtttttctaatttgctcgtggaaacgatagggacgtcatatgg | |
| 2B | pME6010Gib1 | tgagcggatacatatttgaagccgtttttccataggctccgc | 4848 |
| | pME6010Gib4 | tgctcgatgagtttttctaatttgctcgtggaaacgatagggacgtcatatgg | |

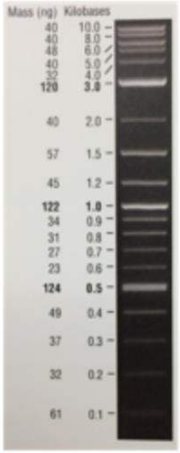
We ran these 4 PCR reactions as follows using the NEB Q5 PCR protocol:

| PCR reaction | Annealing temperature / °C | Extension time / seconds |
|---|---|---|
| 1A | 70 | 30 |
| 1B | 60 | 30 |
| 2A | 70 | 150 |
| 2B | 70 | 150 |

A 0.8% agarose gel was used for the extraction as this offered good separation around 1kb and 5kb. This gel was run and the bands extracted according to our QIAquick Gel Extraction Protocol using NEB purple loading dye and 2-log purple ladder and QIAquick extraction kit. Gel obtained:

lane 1: 2 log purple ladder
lane 2: -
lane 3: 1A
lane 4: 2A
lane 5: -
lane 6: 1B
lane 7: 2B
lane 8: -





# Week 1 continued...

| PCR product | Gel cut-out volume / µl | Concentration of first elution with 20µl EB / ng/µl | Concentration of second elution with 20µl EB / ng/µl |
|---|---|---|---|
| 1A | 200 | 53.7 | 22.8 |
| 1B | 170 | 22.4 | 9.3 |
| 2A | 190 | 41.8 | 26.1 |
| 2B | 180 | 17.1 | 8.1 |

As we later found out these NanoDrop readings are false since the QG buffer in the QIAGEN gel extraction kit interferes with the UV/Vis readings. Following extraction of our PCR products from the gel we used the NEB Gibson Assembly protocol to run an 8hr reaction over night. We ran an 'A' reaction which will insert the KanR gene into the pME6010 plasmid with the native promoter and a 'B' reaction that will insert the KanR gene with a Pamp promoter and optimised RBS.

| | Reaction A | Reaction B |
|---|---|---|
| PCR fragment 1 (fragment) / µl | 1.04 (0.093 pmol) | 2.53 (0.0942 pmol) |
| PCR fragment 2 (vector) / µl | 2.4 (0.031 pmol) | 5.85 (0.0314 pmol) |
| Gibson Assembly MM 2X / µl | 5 | 10 |
| dH2O / µl | 1.56 | 1.62 |
| TOTAL VOLUME / µl | 10 | 20 |

The volumes were chosen to satisfy 100ng vector with a 3-fold increase in the amount of insert. The insert amount must lie between 0.02 and 0.5 pmol and the total volume of total fragments cannot exceed 10µl. Following an overnight 8hr Gibson Assembly the reaction volumes were treated with Dpn1 restriction enzyme that cuts bacterial (methylated) DNA. We transformed the Gibson products into chemically competent DH5-alpha cells as well as into NEB alpha-5 cells. Unfortunately no colonies grew on a KanR plate! We know that the PCR products are correct so we think an issue may have arisen during the Gibson Assembly stage so we will re-do this part next week.

# Week 2

**Tasks completed:**

- Transformed all of Gibson product from week 1 into electro-competent E.coli cells
- Re-ran Gibson assembly but with equimolar amounts of insert:vector.
- Re-ran NEB gibson control to test if the NEB stocks are working.
- Successfully swapped resistance gene for plasmid pME6010 from tetracycline to kanamycin.

**Notebook**:

To run equimolar amounts of insert to vector the following table was calculated:

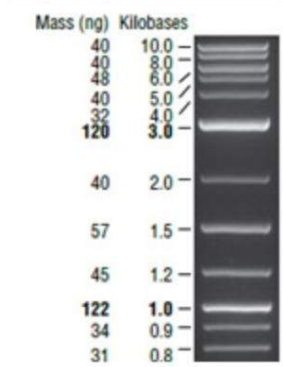
| Reaction identity | Description | Volume of fragments added /µl | Volume MM / µl | Volume Water / µl | Total Volume: µl | Single colonies? |
|---|---|---|---|---|---|---|
| 1 | A - with Karl Gibson mix | 5 | 15 | 0 | 20 | No |
| 2 | B - with Karl Gibson mix | 5 | 15 | 0 | 20 | No |
| 3 | A - NEB MM re-try | 5 | 10 | 5 | 20 | No |
| 4 | B - NEB MM re-try | 5 | 10 | 5 | 20 | No |
| 5 | A - NEB MM 1st attempt | 3.44 | 5 | 1.56 | 10 | No |
| 6 | B - NEB MM 1st attempt | 8.38 | 20 | 1.62 | 20 | No |

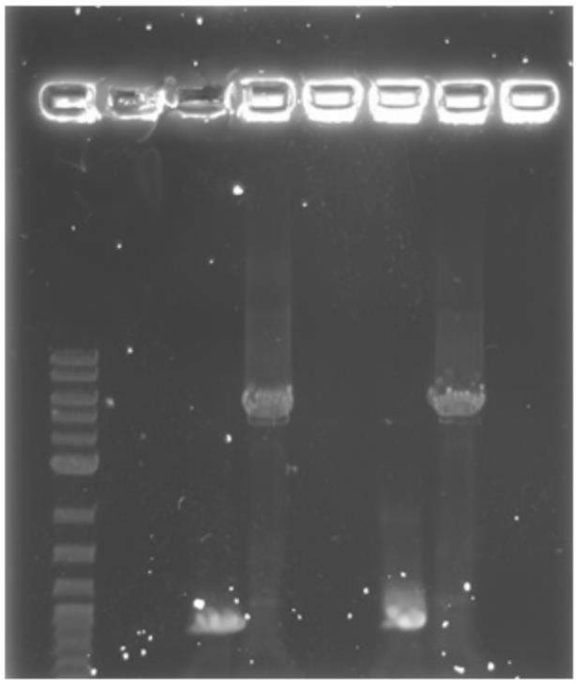
These gibson assemblies were then transformed into E.coli cells using electroporation with the voltage at 1.8kV and the exposure time ranging from 4.8ms to 5.2ms. The cells were then recovered at 37ºC for 1.5 hrs before 100 *µ*l was plated onto KanR plates. These were left overnight at 37ºC.
Unfortunately, there were no colonies grown and as we have no product from our gibson assemblies remaining we will have to repeat the process from Week 1 in order to trouble-shoot the method and get the desired plasmid. It is possible that we accidentally swapped the primers or PCR products around at some point and thus we had 1A with 2B and 1B with 2A in the Gibson assembly. These combinations are not complementary and thus the Gibson would fail meaning that none of our bacteria received intact Kanamycin resistance in the transformation. However there are many other reasons why the process may have failed therefore we went through the entire method trying to cover all instances where we may have made an error.
First we confirmed that the primer sequences on the delivery tubes matched what we had ordered. We ran the PCR as before; we re-calculated the annealing temperatures and extension times with the same result as last week.
In week one, we did the DpnI digest after the Gibson assembly and not directly after the PCR. This time we did the digest immediately after the PCR to ensure that all template DNA was destroyed before running on the gel,

# Week 2 continued...

ensuring cleaner bands on our gel and less contamination from non-PCR products.
We loaded 20 µl of each PCR product onto a 0.8% agarose gel as before. The resulting bands were visualized after staining as shown below:

lane 1: 2 log purple ladder
lane 2: -
lane 3: 1A
lane 4: 2A
lane 5: -
lane 6: 1B
lane 7: 2B
lane 8: -



The bands are not as bright as in the first attempt probably due to less time spent in the ethidium bromide staining tank. The bands correspond with the correct sized fragments as before. This suggests there was no error in the PCR reaction with the exception of again mixing up the primers (the PCR would be successful with any combination of the primers for a 1 and 2 reaction). The bands are cleaner allowing us to try an alternative to the gel extraction in case that caused our assembly and transformation to fail.

Next we excised and extracted our bands from the gel with the same QIAquick Gel Extraction protocol as before but with two changes. First we allowed additional drying time after the step involving ethanol supplemented buffer in case ethanol contamination inhibited the enzymes used in the Gibson assembly later in the process. We also replaced the 20 µl EB buffer with 20µl of MilliQ water. We did our second elution with the kit EB buffer. We then measured the concentration of DNA in each eluted sample using the nanodrop. This still gives us a reasonable idea (despite the interference from the remaining QG buffer residue) as to the amount of DNA in each sample from which to calculate our dilutions for the Gibson assembly reaction.

# Week 2 continued...

| Sample identity | DNA concentration in water eluted sample (ng/µl) | DNA concentration in EB buffer eluted sample (ng/µl) |
|---|---|---|
| 1A | 21.3 | 2.3 |
| 2A | 13.4 | 2.9 |
| 1B | 42.7 | 6.4 |
| 2B | 30.1 | 15.4 |

We used the remaining 10µl of our PCR products to do a Promega PCR clean-up and eluted using water. We gathered the following Nanodrop data:

| Sample identity | DNA concentration in water eluted sample (ng/µl) |
|---|---|
| 1A | 75.4 |
| 2A | 57.3 |
| 1B | 80.5 |
| 2B | 66.3 |

Next we ran the following Gibson assembly reactions:

| Reaction identity | Clean-up method/master-mix used | Volume of Insert, 1 (µl) | Volume of Vector, 2 (µl) | Volume of $dH_2O$ (µl) | Volume of Master-mix (µl) | Volume of ligase (µl) | Total reaction volume (µl) |
|---|---|---|---|---|---|---|---|
| 1 (A) | Gel extract/CK | 0.44 | 3.70 | 0.86 | 13 | 2 | 20 |
| 2 (B) | Gel extract/CK | 0.44 | 3.32 | 1.24 | 13 | 2 | 20 |
| 3 (A) | Gel extract/MM | 0.44 | 3.70 | 5.86 | 10 | - | 20 |
| 4 (B) | Gel extract/MM | 0.44 | 3.32 | 6.24 | 10 | - | 20 |
| 5 (A) | Promega/CK | 0.50 | 2.50 | 2.00 | 13 | 2 | 20 |
| 6 (B) | Promega/CK | 0.46 | 3.02 | 1.52 | 13 | 2 | 20 |
| 7 (A) | Promega/MM | 0.50 | 2.50 | 7.00 | 10 | - | 20 |
| 8 (B) | Promega/MM | 0.46 | 3.02 | 6.52 | 10 | - | 20 |

CK = Ciaran's Mastermix, MM = NEB Gibson Assembly Mastermix

Next we transformed the Gibson assembly products into chemically competent E.coli cells. We decided to run a positive control for each kind of cell used using the pUC19 plasmid provided with the cells. Unfortunately we were limited with the amount of cells available and had to use two different cell strains; this resulted in some variation in the length of the first incubation with the DNA on ice as we began the transformation in the morning but then decided to pause the process so that the cells would be plated out later in the

# Week 2 continued...

day in order to not have too much growth overnight. The NEB 5-alpha cells were also on ice slightly longer before the DNA was added.

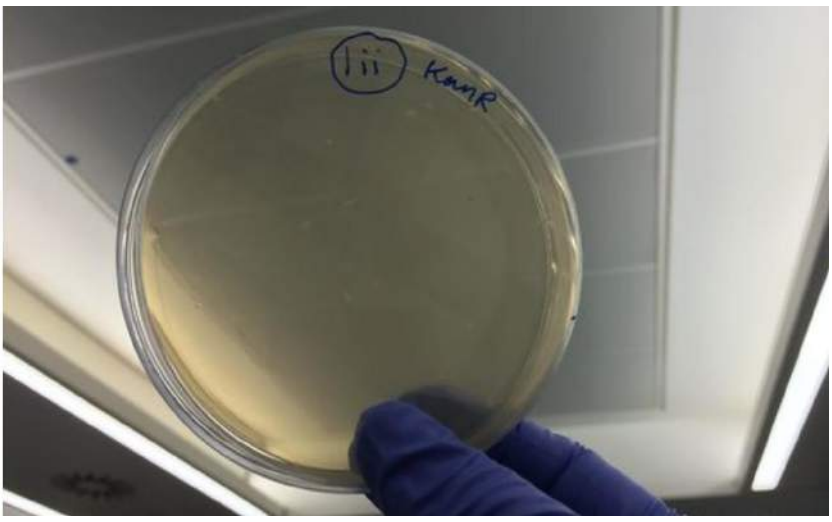
| Sample identity | Cell strain | Length of first incubation on ice |
|---|---|---|
| 1 | NEB 5-alpha | 4 hrs |
| 2 | NEB 5-alpha | 4 hrs |
| 3 | NEB 5-alpha | 4 hrs |
| 4 | NEB 5-alpha | 4 hrs |
| 5 | NEB 5-alpha | 4 hrs |
| 6 | NEB 5-alpha | 4 hrs |
| 7 | NEB 5-alpha | 4 hrs |
| 8 | NEB DH5-alpha | 30 mins |
| 5-alpha control | NEB 5-alpha | 4 hrs |
| (#) DH5-alpha control | NEB DH5-alpha | 30 mins |

The cells were plated out after 1.5 hour incubation. It was noted that one of the sample appeared to have leaked from the Eppendorf into the petri dish containing all the samples in the incubator. We think it was the 5-alpha control as this was the only sample that appeared to have lost volume. Samples 1-8 were plated on Kanamycin plates (i) that we made up during the final incubation period. The remaining sample was re-suspended in ~100µl of SOC to create a concentrated solution of cells that were also plated out on kanamycin plates (ii). The two controls were spread on Ampicillin plates made up by Nick. All plates were placed in the 37ºC incubator overnight. The results in the morning were as follows:

# Week 2 continued...

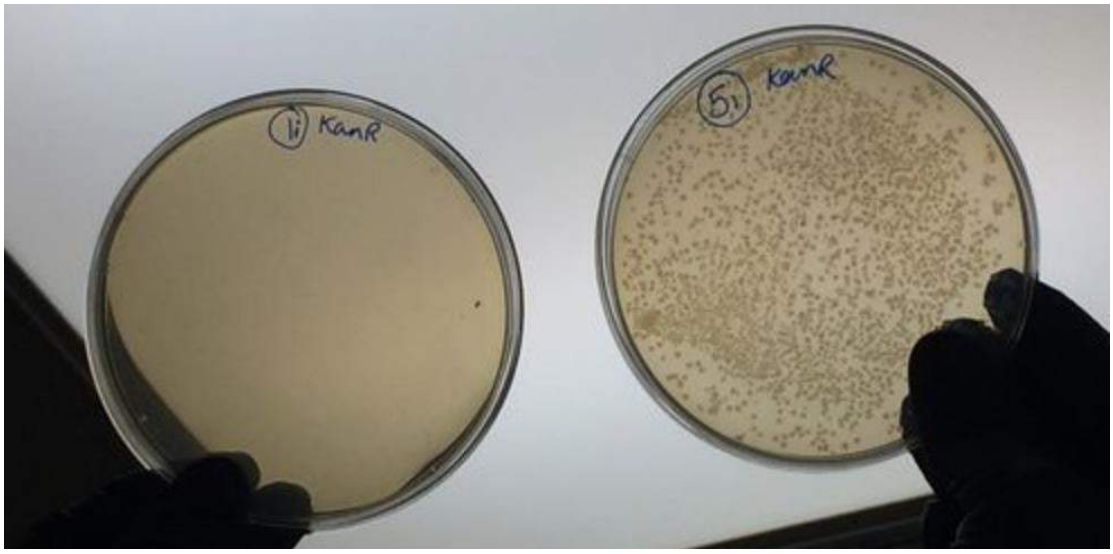
| Sample identity | Cell strain | Clean-up method | Master-mix used | Not concentrated i/ concentrated ii | Growth? |
|---|---|---|---|---|---|
| 1 | NEB 5-alpha | Gel extract | CK | i | - |
| | | | | ii | Y |
| 2 | NEB 5-alpha | Gel extract | CK | i | - |
| | | | | ii | - |
| 3 | NEB 5-alpha | Gel extract | MM | i | - |
| | | | | ii | - |
| 4 | NEB 5-alpha | Gel extract | MM | i | - |
| | | | | ii | - |
| 5 | NEB 5-alpha | Promega | CK | i | Y |
| | | | | ii | Y |
| 6 | NEB 5-alpha | Promega | CK | i | Y |
| | | | | ii | Y |
| 7 | NEB 5-alpha | Promega | MM | i | Y |
| | | | | ii | Y |
| 8 | NEB DH5-alpha | Promega | MM | i | Y |
| | | | | ii | Y |
| 5-alpha control | NEB 5-alpha | - | - | i | Y |
| | | | | ii | Y |
| (#) DH5-alpha control | NEB DH5-alpha | - | - | i | Y |
| | | | | ii | Y |

Apart from the concentrated sample 1ii, none of the samples prepared using the gel extraction protocol grew. Sample 1ii also had limited growth compared to the other plates with colonies:

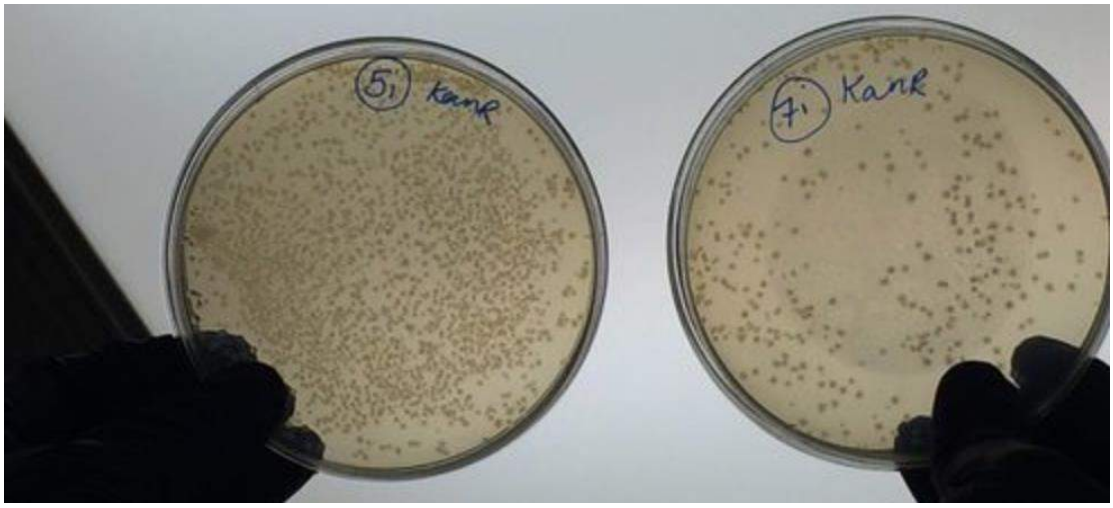


# Week 2 continued...

Samples 1i and 5i differed only in their clean-up method; 5i has lots of growth and 1i has none. This comparison holds true for Samples 2+6, 3+7 and 4+8 with the same result:

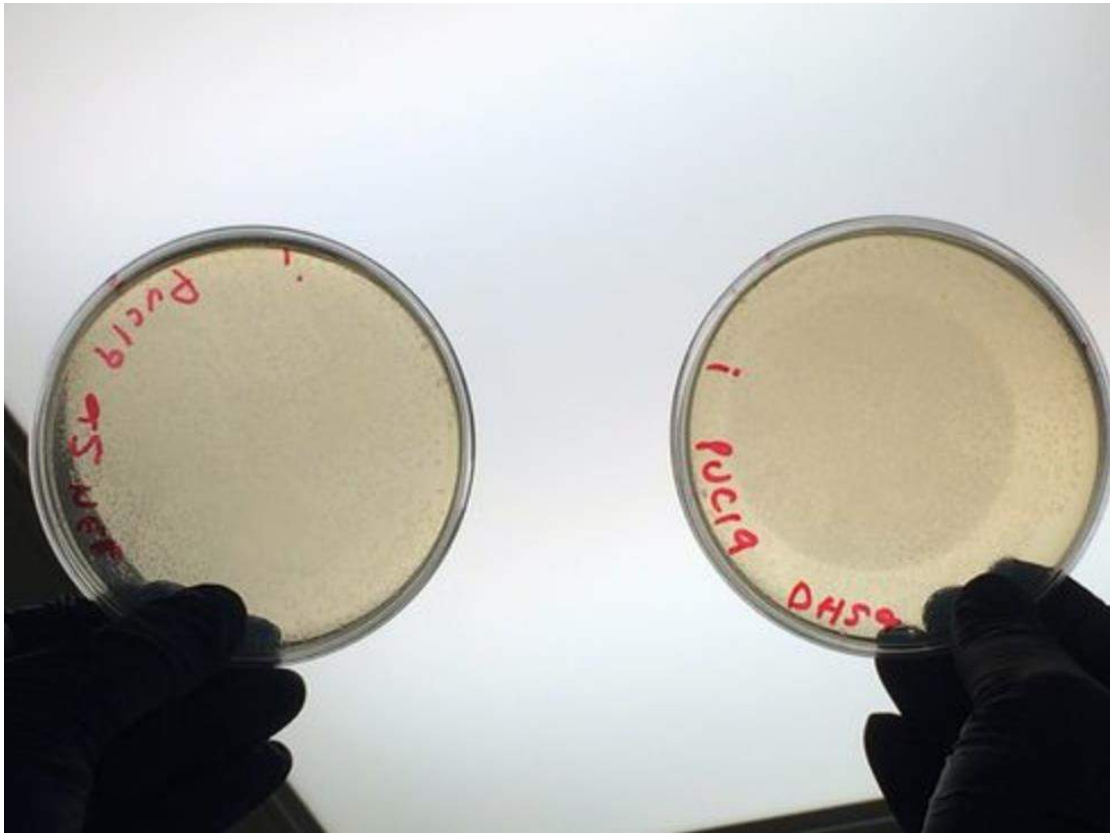



This suggests that we should use the Promega PCR clean-up kit and not the QIAquick Gel extraction. We will still run our samples on a gel to check the quality of our PCR reactions. We may investigate the Promega Gel extraction kit as this will enable us to pick our desired band out of a PCR that has also generated non-specific fragments which we cannot do with the Promega PCR clean-up protocol.
The samples that were treated with the master-mix and ligase supplied by Ciaran had more colonies than those that were treated with the NEB supplied master-mix as shown by the comparison between samples 5i and 7i:



# Week 2 continued...

This suggests that in future we should use the master-mix supplied by Ciaran. All the controls grew showing that the lack of growth of samples 1- 4 was not due to inefficient transformation:




There was no observable difference between sample 8 and sample 7 which were treated the same but which used different chemically competent cells. Thus we can continue to use either the NEB 5-alpha or DH5-alpha chemically competent cells.
There was no observable difference between the samples that correspond to A Gibson assembly reactions (template for PCR primers and therefore fragment = pCM66) and those that correspond to B Gibson assembly reactions (PCR primers and therefore fragment include Pamp).
We picked colonies from the successful plates to grow up cultures overnight:

# Week 2 continued...

| Promoter | Source Plate | Methods | Sample Label |
|---|---|---|---|
| A = Native | 1ii | Gel Extract CK | 1 |
| | 1ii | | 2 |
| | 5i | Promega CK | 3 |
| | 5i | | 4 |
| | 7i | Promega MM | 5 |
| | 7i | | 6 |
| B = $P_{amp}$ | 6i | Promega CK | 7 |
| | 6i | | 8 |
| | 6i | | 9 |
| | 8i | Promega MM | 10 |
| | 8i | | 11 |
| | 8i | | 12 |

We extracted the plasmids from each overnight culture using the Miniprep protocol. We centrifuged 250µl of cells and then added another 250µl and centrifuged in order to get as much DNA as possible. We eluted the each sample in only 30µl of elution buffer to concentrate the sample. We used the Nanodrop to measure the concentration of the plasmid:

| Sample identity | 1st Elution - DNA concentration in eluted sample (ng/µl) | 2nd Elution - DNA concentration in eluted sample (ng/µl) |
|---|---|---|
| 1 | 53.1 | 10.5 |
| 2 | 26.7 | 7.8 |
| 3 | 40.2 | 12.9 |
| 4 | 52.6 | 11.8 |
| 5 | 54.2 | 12.6 |
| 6 | 52.3 | 12.6 |
| 7 | 55.2 | 13.4 |
| 8 | 31.1 | 11.9 |
| 9 | 52.5 | 10.1 |
| 10 | 61.4 | 14.6 |
| 11 | 61.0 | 14.7 |
| 12 | 25.4 | 10.0 |

# Week 3

**Tasks completed:**

- **Amplify pME6010-KanR backbone to linearize for Gibson Assembly**.
- Sequencing data confirms KanR was successfully inserted into our pME6010 plasmid in place of the tetracycline resistance gene.
- First attempt at Gibson Assembly of our gblocks into our vectors.

**Notebook**:
We used PCR to amplify the plasmid backbones of pME6010-KanR (from weeks 1 and 2) and pBBR1 MCS-5 which will host the gblocks for dcmR inducible production and the dcmA upstream region respectively.

| PCR reaction | Template | Primers | Fragment length / bp | Annealing °C | Elong time | PCR extraction used |
|---|---|---|---|---|---|---|
| 1 | pME6010-KanR A (1) | tetRdcmRfullGib1 | 5800 | 70 | 3min | 1i) Gel extraction |
| | | tetRdcmRfullGib2 | | | | 1ii) PCR cleanup |
| 2 | pME6010-KanR B (10) | tetRdcmRfullGib1 | 5800 | 70 | 3min | 2i) Gel extraction |
| | | tetRdcmRfullGib2 | | | | 2ii) PCR cleanup |
| 3 | pBBR1 MCS-5 | pdcmAsfGFPvector1 | 4400 | 63 | 2min 15s | 3i) Gel extraction |
| | | pdcmAsfGFPvector2 | | | | 3ii) PCR cleanup |

We ran the PCR product on a 0.8% agarose gel according to our Gel Electrophoresis protocol and bands were visualised using EtBr staining.

The gel showed bands of unexpected lengths. To test whether this could be because of unexpected DNA fragments being present we will digest each of our PCR templates with a uniquely cutting restriction enzyme: HindIII. With this, we would expect to see lengths of 5.6kb (pME6010-KanR) and 4.8kb (pBBR1 MCS-5). However, if we see a fragment of 2.6kb this means that the template plasmid, pCM66, from which we extracted KanR is still present. Its

# Week 3 continued...

presence would have given a false positive result when grown on kanamycin plates. The resulting gel is shown below.

We then attempted a number of different PCR reactions (see table below) to try and obtain better results. Based on the restriction enzyme digest we will not continue with trying to PCR from the pME6010-KanR B (10) template DNA:

- #5 and #6: We repeated the previous PCR reaction, and again at a lower annealing temperature to encourage initial annealing.
- #7: PCR of other plasmid extraction from KanR plate 1ii (extraction 2 above).
- #8: PCR of the pBBR1 MCS-5 (pSRKGm) template was repeated but at a higher temperature after a second annealing site was seen when the hybridisation parameters were set to a lower less stringent temperature in

# Week 3 continued...

SnapGene.

The PCR reaction volumes were as follows (total reaction volume = 25µl):

The PCR Gel is seen below. From this we can see that lowering the annealing temperature for plasmid 1 worked very well with an expected band of 5.8kb while the chase PCR (5) and plasmid 2 (7) did not work as well. The pSRK

Gm (pBBR1-MCS5) plasmid PCR worked relatively well with a small band indicated. However, this was a weak band amongst some other, non-specific, bands.

# Week 3 continued...

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Marked bands were extracted using the QIAGEN PCR gel extraction. We also performed a Promega PCR clean-up on the remaining 15$\mu$l of the loading DNA. These clean-ups eluted the following concentrations:

Using these concentration values we set up the Gibson assembly reactions for each plasmid to insert the gblocks. Into pME6010-KanR goes RBSdcmRnew (fragment 1), ptetW****r (fragment 2), and pamptetRterm (fragment 3). Into plasmid pSRK-Gm goes pdcmAsfGFP (fragment 1). The Gibson reaction was set up to have the amount of vector between 30-100ng and the fragments in equimolar amounts with the total DNA volume not exceeding 5$\mu$l. To this was added 13$\mu$l Gibson Master Mix and 2$\mu$l Taq Ligase to make a total reaction concentration of 20$\mu$l.

These Gibson reactions were set up for 8hrs overnight at a temperature of 50ºC. Plasmid selection for PCR ID #6 was based upon sequencing data from Source Biosciences, Oxford using our 15A sequencing primer. This sequencing data shows the 6 candidate plasmids extracted from the KanR plates.

We treated the resulting Gibson assembly products with a PCR clean-up (labelled resulting samples 6i and 8i respectively) and gel extraction (labelled resulting samples 6ii and 8ii respectively). We then transformed these samples into chemically competent NEB alpha-4 cells. Having allowed these to grow up overnight on kanamycin plates we had colonies on all four plates. This confirms that the transformation worked but does not confirm that our Gibson assembly reaction was successful. We will test this next week so the plates have been placed in the cold room to prevent too much growth.

# Week 4

---

**Tasks completed:**

- **Confirmed that Gibson assembly had not worked by sequencing and restriction enzyme digests**
- PCR amplified backbones of constructs to re-do Gibson assembly
- Made frozen stocks of chemically competent E. coli MG155 cells

We grew up overnight liquid cultures sampled form colonies of cells transformed with Gibson assembly reaction products. We extracted the plasmids from these samples (in order to get as much DNA as possible we used all the cells available resulting in several centrifugation steps to achieve a bacterial pellet before lysis). We eluted the each sample in only 30µl of elution buffer to concentrate the sample and the following NanoDrop data was acquired:

All samples were prepared and sent for sequencing by Source Bioscience. N.B. each sample sent for sequencing was at a lower concentration than the optimal so we provided them with 5µl of the above concentrations. For samples 1-12 we ordered our own sequencing primer that would give a readout covering the backbone and our insert if it has correctly assembled in

# Week 4 continued...

the construct, however this did not arrive in time. Instead we asked Source Bioscience to use a primer that is complementary to a region inside our insert. Our sample was unable to be sequenced which suggesting that the DNA the sequencing primer was complementary to was not in the sample. This indicates that our insert was not in the fragment and thus our Gibson assembly had not worked.

To test this theory we did a double restriction enzyme digest using HindIII and PstI using the following amounts and incubating at $37^0$c for an hour:

We ran these digests on a 0.8% agarose gel, the results of which are shown below:

# Week 4 continued...

All the samples we ran at approximately 5.5kb and were only cut once. This is what we would expect from the backbone without the inserts:

If the construct had assembled correctly we would have expected 2 bands one at ~2kb and one at ~5.5kb:

When our own primer arrived we re-sent samples 1-12 for sequencing with

# Week 4 continued...

the new primer  however these sequencing results were also negative for the insert.

This further confirms our conclusion that our Gibson assembly reaction has failed and the vector backbone re-ligated without the insertion of the

fragments. To ensure that none of our colonies had the complete construct we selected 12 more colonies from plates 6i and 6ii and grew them up in liquid culture with kanamycin overnight (labelled 1B-12B). We then extracted the plasmids from the cultures and repeated the same double restriction enzyme digest with HindIII and PstI. We however found the same result as before with only a single band running at ~5.5kb:

This indicates that none of the colonies on our plate have the full construct but have the re-ligated backbone.

Sequencing data for samples 13-24 which potentially contain the pSRK-pdcmA-sfGFP construct came back as negative for our insert, as shown

# Week 4 continued...

below:

To confirm that none of the colonies on our plates had the correct construct we did a rough colony PCR on 48 colonies. We did not use specially designed primers but they should have produced a defined PCR product if our inserts were in any of the colonies. None of the colonies showed a defined product as shown below, however this could have been due to PCR failure as opposed to the lack of inserts as no control was run:

Taken together, the evidence suggests that the pSRK plasmid backbone had re-ligated during the Gibson assembly without the G-blocks ligating.

# Week 4 continued...

Thus we will have to repeat the Gibson assembly for both our constructs. To this end we have used PCR to amplify the pME6010 Kan and pSRK Gm vector backbones, essentially repeating PCR reactions #6 and #8:

PCR reactions #14 and #15 were prepared as follows:

We ran the PCR products for approximately 90 minutes at 70V on a 0.7% agarose gel, shown below:

The band in lane 3 corresponds to the size of the pME6010KanR backbone at ~5.5kb. The band of size ~4.5kb in lane 5 corresponds to the pSRK Gm backbone. Due to the possibility that the indistinct separation of the large band in lane 3 was in fact two bands, when excising the fragment from the gel this band was cut in half. Thus three gel extractions were undertaken; #14TOP, #14BOTTOM and #15. The samples were eluted in 20µl of water. Following gel extraction the concentrations of DNA were determined using the NanoDrop:

# Week 4 continued...

As the concentrations from the second elution are so much higher, we will use those samples in our Gibson assembly reactions.

We set up the following Gibson reactions. Gibson #2, #4 and #5 have equimolar amounts of vector to inserts. Reactions #1 and #3 are not equimolar. All these reactions were catalysed by the Mastermix and ligase provided by Ciaran's lab.

The products of these Gibson reactions were then transformed into various types of cells (as we had a limited amount of the NEB alpha-5 cells). This will allow us to compare the transformation efficiency of the various types of cells available to us.

# Week 4 continued...

The results of the transformations are shown below. All the transformations grown on Kanamycin plates but transformations h., h.ii. i., i.ii, l., m. and n. should have been on gentamycin plates; thus these transformations were repeated as shown in the second table below.

Plated out new transformations:

Key: See Gibson reactions for construct components, NEB = NEB 5-alpha cells, CK TSB = DH5-α provided by Ciaran, Glen = DH5-α cells made competent by Glen

This suggests we have four colonies that potentially contain our pME6010 KanR construct. This is supported by the fact that the positive control for transformation c.ii has growth and the negative control, whilst it potentially has a single colony has less growth than the c.ii plate and this is also possibly contamination. There is also potentially another colony on plate b.ii. The colonies on plate h.ii are most likely contamination as none of our construct should have grown on the kanamycin – however as there were quite a few colonies on plate m. which also shouldn't have had any growth this potential resistance may be something to bear in mind.

We made up the following overnight liquid cultures (in 5ml LB):

# Week 4 continued...

Cultures c.ii. 1-4 were mini-prepped and eluted in 30µl of EB buffer. The following concentrations were determined by Nanodrop:

The growth on plates p.- r. suggests that the small colonies might contain our pSRK Gm construct . After 16 hours in the incubator the colonies were very small, so the plate p.ii. was left on the bench and liquid cultures made a further 8 hours later. These liquid cultures had no growth the next morning.

We left p.ii (after a short while in the cold room) and the liquid cultures in incubator to see if there was any growth over a longer period of time.

Plates e. f. and g. provide a comparison of transformation efficiency between the three types of competent cells. Plates e. and g. had full lawns of colonies. Plate f. had fewer colonies but still quite a lot. Thus Glen's cells seem to be as good as the NEB cells. Ciaran's do not have as high transformation efficiency.

In addition to the above we made stocks of chemically competent MG155 E.coli cells to transform our plasmids into when we have succeeded in constructing them.

# Week 5

**Tasks completed:**

- **Attempt at Gibson Assembly of pME6010 construct (tetR, dcmR)**
- PCR amplify pSRK Gm plasmid backbone at varying annealing temperatures

To troubleshoot our pME6010 Gibson Assembly we attempted to run a 20 cycle PCR with just our three gblocks (PamptetRterm, RBSdcmRnew, PtetW**ker) followed by 30 cycles with the flanking primers added. The conditions are seen below:

We ran the products on a gel to see if we obtained any product. The gel is seen below:

# Week 5 continued...

As can be seen our attempt at this PCR of our three gblocks failed. This may be because our gblock dilutions had very little DNA in them (lanes 10,11,12 above). Following this failed attempt to anneal our gblocks we decided to troubleshoot our Gibson Assembly. This process began with re-running the amplification of the pME6010 KanR backbone. This was done at annealing temperatures 50, 55, 60, 65, 72 ºC. We repeated this with our two versions of the pME6010 KanR backbone that we made with the native KanR promoter ("1") and with an inserted Pamp (ampicillin promoter) ("10"). This gel showed a strong band at all temperatures for "1" (native KanR promoter). Backbone "10" gave non-specific bands so we went ahead with Gel Extraction of only backbone "1" which gave elution concentrations of 19.3 ng/$\mu$L. Following gel extraction we re-ran the gibson using gblocks straight from the original re-hydration rather than using any previous dilutions. This gibson used 50ng vector and equimolar amounts of the three gblocks and was run for 8hrs overnight at 50ºC. Following this gibson reaction we transformed the product as follows:

This gave correct colony growth for both positive and negative controls. We also achieved growth on plates IV (40 colonies) and VII (6 colonies). We made liquid cultures of 24 colonies (4 from plate VII, and 20 from plate IV). This was followed by a MiniPrep on all of these which yielded the following elution concentrations (ng/$\mu$L):

# Weeks 6-7

We then tried an alternative method to making our two plasmid system involving a restriction enzyme digestion and ligation approach:

1. Amplify the dcmAsfGFP gblock with BamHI restriction sites at both ends.
2. Insert into pOXON-1 after BamHI restriction digest.
3. Amplify dcmR g block with PstI and KpnI sites.
4. Insert into pSRK Gm after digestion with PstI and KpnI (RE sites located in MCS).
5.

As pSRKGm contains the lac promoter we could potentially use this as a way of inducing expression of dcmR instead of the tet operator system and ATC.

However attempts at this approach were unsuccessful despite several attempts and troubleshooting and PCR optimisation so we returned to Gibson assembly.

# Week 8

**Tasks completed:**

- **Successfully made full pOXON-2 dcmR construct, one half of the biosensor system, via Gibson assembly**
- Unsuccessfully attempted to make the full pOXON-2 dcmR construct using a restriction-ligation approach.
- Verified that the ligations from last week had not yielded either construct.
- Attempted to make the pSRKGm pdcmAsfGFP construct, the second half of the biosensor system, via Gibson assembly.
- Unsuccessfully attempted to blunt end ligate pdcmAsfGFP and RBSdcmRnew gblocks into a holding vector

Gibson assembly of pOXON-dcmR

pOXON-2 was mini-prepped from the two liquid cultures made yesterday from cells transformed with pOXON-2 (construct #45) and the following concentrations of DNA obtained:

| **Sample** | **DNA concentration (ng/µl)** |
|---|---|
| #45i | 42.3 |
| #45ii | 34.1 |

| Reaction ID | Template DNA | Primers | Annealing Temperature ($^0$c) | Extension Time |
|---|---|---|---|---|
| 50 | 1/10 dilution of RBSdcmRnew gblock | DcmR Gib2 complement | 72 | 1m |
| | | NcoI site addition 2 | | |
| 51 | #45i | tetRdcmRfullGib 2 | 72 | 3m 30s |
| | | NcoI site addition 1 | | |
| 52 | 1/10 dilution of RBSdcmRnew gblock | DcmR fragment Gib A | 72 | 1m |
| | | DcmR fragment Gib B | | |
| 53 | #45i | tetRdcmRfullGib 2 | 72 | 3m 30s |
| | | DcmR vector | | |

| | | | | |
|---|---|---|---|---|
| | | Gib A | | |

The dcmR g block and the pOXON-2 construct were amplified by PCR using

# Week 8 continued...

the NEB Q5 protocol as shown in the table below. Reactions #50 and #51 will produce dcmR and pOXON-2 with compatible ends for ligation after digestion by NcoI and PstI. Reactions #52 and #53 will be produce dcmR and pOXON-2 with compatible overhangs for a Gibson assembly reaction. This will provide us with two different approaches to inserting the full dcmR gene into the pOXON-2 construct which has an incomplete version of the gene:
The resulting products were run on 1% agarose gel with 1kb plus DNA ladder as shown below:

# Week 8 continued...

The marked bands were of the correct size for the inserts and vectors and we therefore excised them and extracted the DNA using the Promega gel extraction protocol. The following DNA concentrations were obtained via NanoDrop:

| Gel extract | DNA concentration (ng/µl) |
|---|---|
| #50 | 5.2 |
| #51 | 4.9 |
| #52 | 4.7 |
| #53 | 7.7 |

Two 8 hour Gibson assembly reactions were set up as follows:

| Reaction Label | Component | Volume (µl) |
|---|---|---|
| Exp | 30ng vector – gel extracted #53 | 3.9 |
| | Equimolar amount of insert – gel extracted #52 | 0.8 |
| | MilliQ water | 0.3 |
| | Karl's experimental Mastermix (incl. ligase) | 15 |
| Com | 30ng vector – gel extracted #53 | 3.9 |
| | Equimolar amount of insert – gel extracted #52 | 0.8 |
| | MilliQ water | 0.3 |
| | Commercial Mastermix | 13 |
| | Ligase | 2 |

**(Components were added in ice with ligase (where appropriate) added last. PCR machine was heated to $50^0$c before samples were placed inside.)**

The product of the Gibson assembly reactions using each Mastermix were transformed separately into chemically competent E.coli cells. 1µl of pOXON-

1 was transformed as a positive control, and 1µl of the gel extracted #53 as a negative control (linearized pOXON-1 with Gibson assembly overhangs). Colony PCR of colonies taken from the plates produced a positive result suggesting that we have succeeded in making the pOXON-2 dcmR construct.

The positive colonies were sent for sequencing which confirmed the colony PCR result.

We transformed the sequenced construct into chemically competent MG155 E.coli and spread on kanamycin plates using the same protocol as our chemically competent DH5α E.coli. The plate was incubated overnight at $37^0$c.

Restriction-ligation of pOXON-2-dcmR

The gel extracted products of PCR reactions #50 and #51 were used in the following restriction digests and ligation reaction.

# Week 8 continued...

To generate the ligation inserts the following 40µl reaction was incubated for 2.5 hours at $37^0$c:

| **Component** | **Volume (µl)** |
|---|---|
| DNA (dcmR) | 28 |
| NEB RE Buffer 3 | 4 |
| PstI | 1 |
| NcoI | 1 |
| BSA (100x) | 0.4 |
| MilliQ water | 5.6 |

To generate the vector backbone the following 10µl reaction was incubated for 2.5 hours at $37^0$c:

| **Component** | **Volume (µl)** |
|---|---|
| DNA (pOXON-2) | 3 |
| NEB RE Buffer 3 | 1 |
| PstI | 0.5 |
| NcoI | 0.5 |
| BSA (100x) | 0.1 |
| MilliQ water | 4.9 |

The products of these reactions were treated with Promega PCR clean-up (as both had already been run on gel to select the correct pieces of DNA by size) and the following concentrations of DNA were obtained:

| **Sample** | **DNA concentration (ng/µl)** |
|---|---|
| Digested dcmR | 6.8 |
| Digested pOXON-2 | 6.5 |

As the concentrations of DNA were low instead of calculating an exact 3:1 insert: vector molar ratio the following ligation reaction was set up with a 3:1 volume ratio and incubated overnight at $16^0$c:

| **Component** | **Volume (µl)** |
|---|---|
| Digested pOXON-2 | 10 |
| Digested dcmR | 30 |
| T4 DNA ligase | 1 |
| T4 DNA ligase buffer | 5 |
| MilliQ water | 4 |

A second ligation reaction was designed as negative control but was only run for three hours.
The product of both ligation reactions were transformed into chemically competent DH5α cells.

The transformation failed therefore a flanking PCR was done using the ligation product in order to determine if the ligated construct had the correct

# Week 8 continued...

insert; this produced a negative result suggesting the ligation had failed.

Gibson assembly of pSRKGm pdcmAsfGFP

In order to generate the backbone for Gibson assembly of pSRKGm pdcmAsfGFP we conducted the following PCR reactions to linearize the pSRKGm vector backbone with appropriate overhangs. Calculated annealing temperatures for the primers used was $65^0$c however, two other temperatures were also tested. New dilutions of primers were used:

| Reaction ID | Template DNA | Primers | Annealing Temperature ($^0$c) | Extension Time |
|---|---|---|---|---|
| 58 | pSRK Gm (pBBR1 MCS-5) | pdcmAsfGFP vector 1 | 60 | 2m 30s |
| | | pdcmAsfGFP vector 2 | | |
| 59 | pSRK Gm (pBBR1 MCS-5) | pdcmAsfGFP vector 1 | 65 | 2m 30s |
| | | pdcmAsfGFP vector 2 | | |
| 60 | pSRK Gm (pBBR1 MCS-5) | pdcmAsfGFP vector 1 | 70 | 2m 30s |

| | | | | |
|---|---|---|---|---|
| | | pdcmAsfGFP vector 2 | | |

PCR products were run on a 1% agarose gel with 1kb plus DNA ladder with the following result:

# Week 8 continued...

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The three bands at ~4.3kb (predicted size of linearized vector) were excised and extracted together (using the Promega, not QIAgen protocol) with a final concentration of 10.4ng/µl.

Prepared the following Gibson assembly reaction:

| **Component** | **Volume (µl)** |
|---|---|
| 25ng vector – pSRK Gm linearized with Gibson overhangs | 2.4 |
| Equimolar amount of insert – pdcmAsfGFP gblock | 1.8 |
| MilliQ water | 0.8 |
| Experimental Mastermix (no | 15 |

| | |
|---|---|
| additional ligase needed, provided by Karl) | |

We also intended to prepare a second assembly reaction with different ratios of vector to insert but we discovered we had little gblock remaining. We need to PCR amplify the gblock before using it again.

We transformed 10µl of the Gibson reaction products into chemically competent DH5α E.coli and spread in gentamycin plates. 1µl of the stock pSRK Gm vector was transformed as a positive control and 1µl of the pSRK Gm vector linearized with overhangs for Gibson assembly as a negative control. All plates were incubated at $37^0$c overnight.

The next day there was no growth on the negative control and lots of growth on the positive control. Initially there were no colonies visible on the construct plates but after 5 hours more incubation there were 3 small colonies. These shall be tested for the presence of the construct by colony PCR.

<u>Blunt end ligation of gblocks into holding vector</u>

Mini-prepped the liquid cultures made by Karl selected on the basis of colony PCR. The following concentrations were obtained and the DNA sent for sequencing with Primer 1 (supplied by Karl, primer 685):

| **Sample** | **Concentration (ng/µl)** |
|---|---|
| dcmR2 | 133.9 |
| dcmR3 | 144.1 |
| dcmR4 | 118.1 |
| sfGFP1 | 123.2 |
| sfGFP2 | 120.9 |
| sfGFP3 | 122.8 |

The sequencing data for the blunt end ligations of the g blocks confirmed that the g blocks had not been inserted into the holding vector.

# Week 9-14

**Tasks completed:**

- **Successfully made a construct containing PdcmA-sfGFP.**
- Transformed cells with different combinations of our complete constructs to allow characterisation of DcmR and biosensor construction.

Attempts to continue constructing the pSRKGm-PdcmA-sfGFP failed. However, cloning via Gibson assembly into a different vector, pJ404, was successful. This plasmid is suitable for expression E.coli and since it contains a pBR322 origin of replication it is compatible with pOXON-2-dcmR-mCherry.

Since DcmR is predicted to regulate expression of DcmA as well as auto-regulating its own expression, we decided to insert this promoter-containing intergenic region in both orientations upstream of sfGFP. This means we have two constructs:
- One with sfGFP in a position corresponding to the equivalent position of dcmA (labelled as 'forward' or PdcmA)which can express sfGFP under the PdcmA promoter.
- A second construct with sfGFP in the equivalent position of dcmR (labelled as 'reverse' or PdcmR) that can express sfGFP under the promoter PdcmR.
The following combinations of components were introduced into DH5α. Pdcma was introduced via transformation with the PJ404-pdcmA-sfGFP construct; PdcmR via the PJ404-pdcmR-sfGFP construct; dcmR via our pOXON-dcmR-mCherry construct to generate the following cultures:

| | **Cells** | **Containing components** | **Antibiotic Resistance** |
|---|---|---|---|
| A | DH5 | PdcmA only | Ampicillin |
| B | DH5 | PdcmA + dcmR | Ampicillin/Kanamycin |
| C | DH5 | PdcmR only | Ampicillin |
| D | DH5 | PdcmR + dcmR | Ampicillin/Kanamycin |
| E | MG1651 | - | - |

# Week 15

**Tasks completed:**

- **Characterised the action of DcmR on both PdcmA and PdcmR.**
- Undertook experiments to determine effect of DCM on this system.

**Media recipes**

M9 minimal media

| Component | Volume | |
|---|---|---|
| MilliQ water | 36ml | |
| 1 in 10 M9 | 4ml | |
| 50% glycerol | 400µl | |
| 1% thiamine | 40 µl | (1 in 1,000) |
| 1M CaCl | 4 µl | (1 in 10,000) |
| 1M $MgSO_4$ | 80 µl | (1 in 500) |
| Total volume | ~40ml | |

EZ Rich Defined media

| Component | Volume |
|---|---|
| 10x MOPS mixture | 5ml |
| 0.132M $K_2HPO_4$ | 0.5ml |
| 10x ACGU | 5ml |
| 5x supplement EZ | 10ml |
| Sterile $H_20$ | 29ml |
| 0.5% glycerol | 0.5ml |
| Total volume | 50ml |

Glycerol has been used instead of glucose to avoid possible interference at the promoters due to CRP binding.

**Experiment to determine action of DcmR**

- 1ml of each of the overnight cultures of A-E were pelleted, LB removed, washed and resuspended in 1ml M9 minimal media (see recipe above).
- The following 1/100 subcultures were made:

| | Volume |
|---|---|
| Cells suspension | 40 µl |
| Antibiotics | 4µl of each required |
| M9 minimal media | 4ml |
| ATC (to induce dcmR expression) | (final concentration = 100ng/ml) |

- 200µl samples of each subculture were added to a 96 well plate as follows:

# Week 15 continued...

| Sample | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M9 | A | | | | | | | | | | | | |
| E | B | | | | | | | | | | | | |
| A | C | | | | | | | | | | | | |
| B | D | | | | | | | | | | | | |
| C | E | | | | | | | | | | | | |
| D | F | | | | | | | | | | | | |
| | G | | | | | | | | | | | | |
| | H | | | | | | | | | | | | |

- Plate was placed in the Tecan plate reader (incubated at 37$^0$c, shaken). Fluorescence and OD readings were taken every 15minutes for 16hours.

**Experiment to determine action of DcmR - repeat**

- 1ml of each of the overnight cultures of A-E were pelleted, LB removed, washed and resuspended in 1ml M9 minimal media (see recipe above).
- The following 1/50 subcultures were made:

| | Volume |
|---|---|
| Cells suspension | 80 µl |
| Antibiotics | 4µl of each required |
| M9 minimal media | 4ml |
| ATC (to induce dcmR expression) | 4 µl (final concentration = 100ng/ml) |

- 200µl samples of each subculture were added to a 96 well plate as follows:

| Sample | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M9 | A | | | | | | | | | | | | |
| E | B | | | | | | | | | | | | |
| A | C | | | | | | | | | | | | |
| B | D | | | | | | | | | | | | |
| C | E | | | | | | | | | | | | |
| D | F | | | | | | | | | | | | |
| | G | | | | | | | | | | | | |
| | H | | | | | | | | | | | | |

- Plate was placed in the Tecan plate reader (incubated at 37$^0$c, orbital shaken). Fluorescence and OD readings were taken every 15minutes for 4hours.

The data from both of these repeats is shown on our wiki results page.

# Week 15 continued...

**Experiment to determine effect of DCM on the system**

- 1 in 11 sub-cultures of the remaining cell suspensions (2x of each A-E) from the previous experiment were made in EZ rich defined media (see above recipe).

| | Volume |
|---|---|
| Cells suspension | 450µl |
| Antibiotics | 5µl |
| EZ rich defined media | 5ml |
| ATC (to induce dcmR expression) | 5 µl (final concentration = 100ng/ml) |

- Subcultures were incubated in 50ml falcon tubes in the shaker at $37^0$c for 4 hours.
- 10µl of DCM was added to one of each A-E subculture to give a final [DCM] ~30mM
- Fluorescence was measured after 2hrs in cuvettes in the fluorimeter.
- 200 µl of each subculture was added to a 96 well plate as shown below:

| Sample | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EZ only | A | | | | | | | | | | | | |
| E | B | | | | | | | | | | | | |
| A | C | | | | | | | | | | | | |
| B | D | | | | | | | | | | | | |
| C | E | | | | | | | | | | | | |
| D | F | | | | DCM | DCM | DCM | | | | DCM | DCM | DCM |
| | G | | | | DCM | DCM | DCM | | | | DCM | DCM | DCM |
| | H | | | | DCM | DCM | DCM | | | | | | |

- Unfortunately the data obtained was not usable due to a fault in the plate reader and so we were unable to use it to observe if DCM has an effect on our system.

# LAB BOOK

## Bioremediation

# Weeks 1 & 2

**Tasks completed:**

- Purified the pUNI-ABTUNJK and the pSB1C3-ABTUNJK plasmids
- Tested the antibiotic resistance of both Pseudomonas strains
- Successfully transformed pUNI-ABTUNJK into P. putida

**Notebook:**

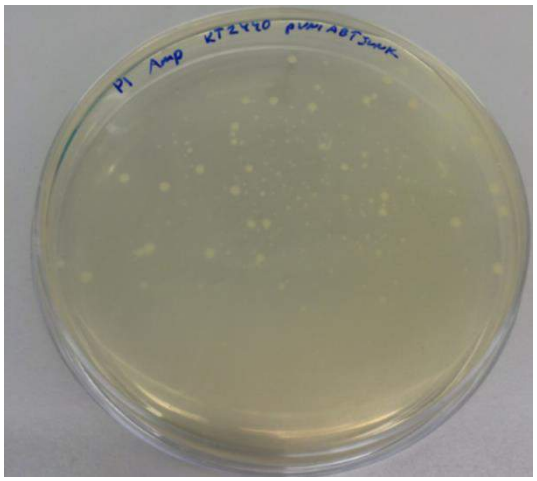
We grew bacteria containing pUni-ABTUNJK (1) and pSB1C3-ABTUNJK (2) in LB overnight. The bacteria were pelleted by centrifugation (twice, to make sure there is no medium left during the purification steps). We used the Miniprep kits to extract each plasmid and then measured the DNA concentration using Nanodrop with the following results: 58 ng/$\mu$L for pUni-ABTUNJK, and 142.1 ng/$\mu$L for pSB1C3-ABTUNJK

In order to transform the plasmids we will be working with into Pseudomonas, we have generated liquid cultures of both Pseudomonas putida (KT2440)and Pseudomonas fluorescens (SBW25).

The next day, we have transformed the three plasmids listed below into both strains using the electroporation protocol for Pseudomonas.

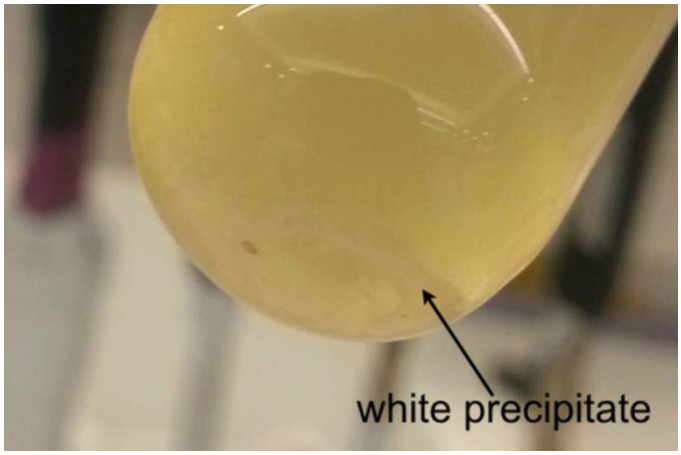
| Acronym | Strain | Plasmid | Resistance |
|---|---|---|---|
| P1 | Pseudomonas putida | pUNI-ABTUNJK | Ampicillin |
| F1 | Pseudomonas fluorescens | pUNI-ABTUNJK | Ampicillin |
| P2 | P. putida | pME6010 | Tetracycline |
| F2 | P. fluorescens | pME6010 | Tetracycline |
| P3 | P. putida | pSRKGm | Gentamicin |
| F3 | P. fluorescens | pSRKGm | Gentamicin |

We have collected the plates from the previous day and found that there was bacterial growth on all plates. However, the Pseudomonas fluorescens strain formed a big bacterial lawn on the ampicillin-containing plate, which could potentially suggest that Pseudomonas fluorescens is inherently resistant to ampicillin. The most important result is the fact that we managed to transform the pUNI-ABTUNJK plasmid into P. putida, especially since pUNI is not a plasmid designed for Pseudomonas strains.



# Weeks 1 & 2 continued...

We have taken aliquots of the liquid cultures to generate frozen cultures according to our standard protocol. However, we found a white precipitate in the liquid culture of P1 (Pseudomonas putida with the pUNI-plasmid). To make sure that this is not a contamination we generated five liquid cultures of P1 from the original plate to grow them overnight. Again, in all five replicates, we could see a white precipitate:




We are not entirely sure why the white precipitate forms, but a possibility is that the plasmid causes a stress response of the cell. Alternatively, the protein might not be expressed, processed, or folded properly, leading to its aggregation.

To test whether our Pseudomonas fluorescens strain is actually resistant to ampicillin, and whether there are any other resistances, we plated out both WT-strains on ampicillin, gentamicin, kanamycin, tetracycline and chloramphenicol-plates. In fact, the next day, we found Pseudomonas fluorescens growing on the ampicillin-plate and Pseudomonas putida growing on the chloramphenicol plate.

So far we have inserted the pUNI-plasmid containing the subunits for the microcompartment into P. putida. However, in order to test whether these subunits are in fact expressed in P. putida we prepared a western blot by making a RAPID reducing buffer and a Blotting Buffer. We spun down 5mL aliquits of the liquid cell culture for 10 minutes at 2000rpm. After removing the supernatant, we resuspended the the pellet in the SDS-loading buffer. After freezing at -20°C for one hour and then boiling for 10 min. (to denature the proteins), we stored the samples ready for the Western blot in the -20°C freezer.

# Week 3

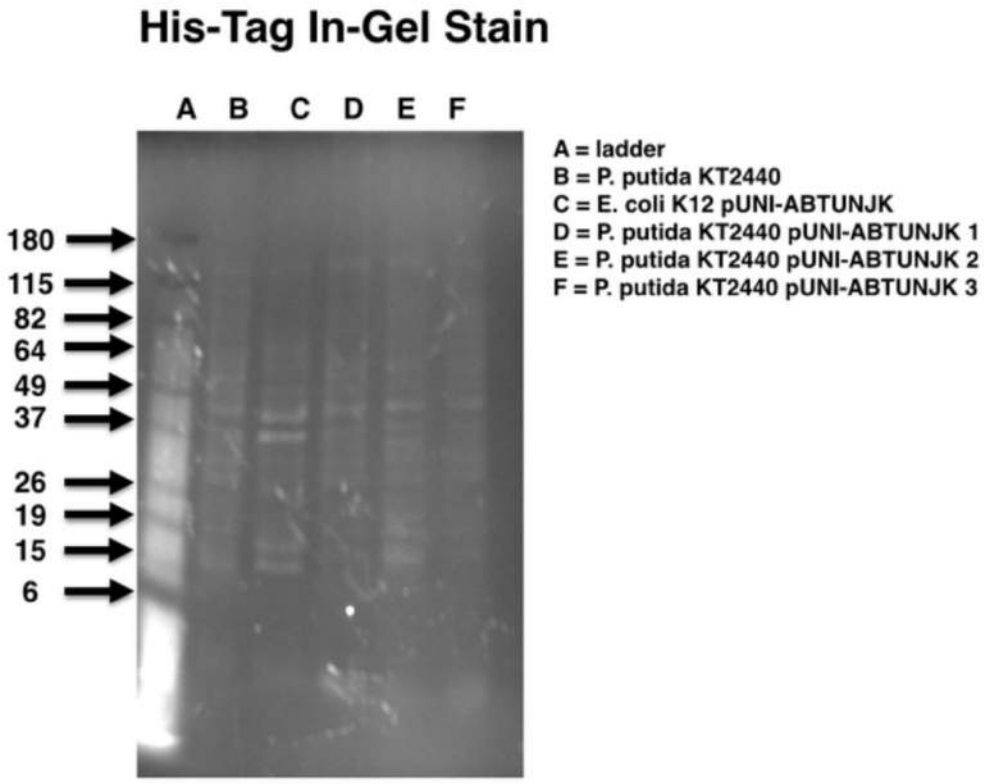
**Tasks completed:**

- Primer design for insertion of wild-type dcmA into M. extorquens DM4
- His-Tag In-Gel Stain of P. putida transformed with pUNI-ABTUNJK plasmids
- Western blot of P. putida transformed with pUNI-ABTUNJK plasmids

**Notebook:**

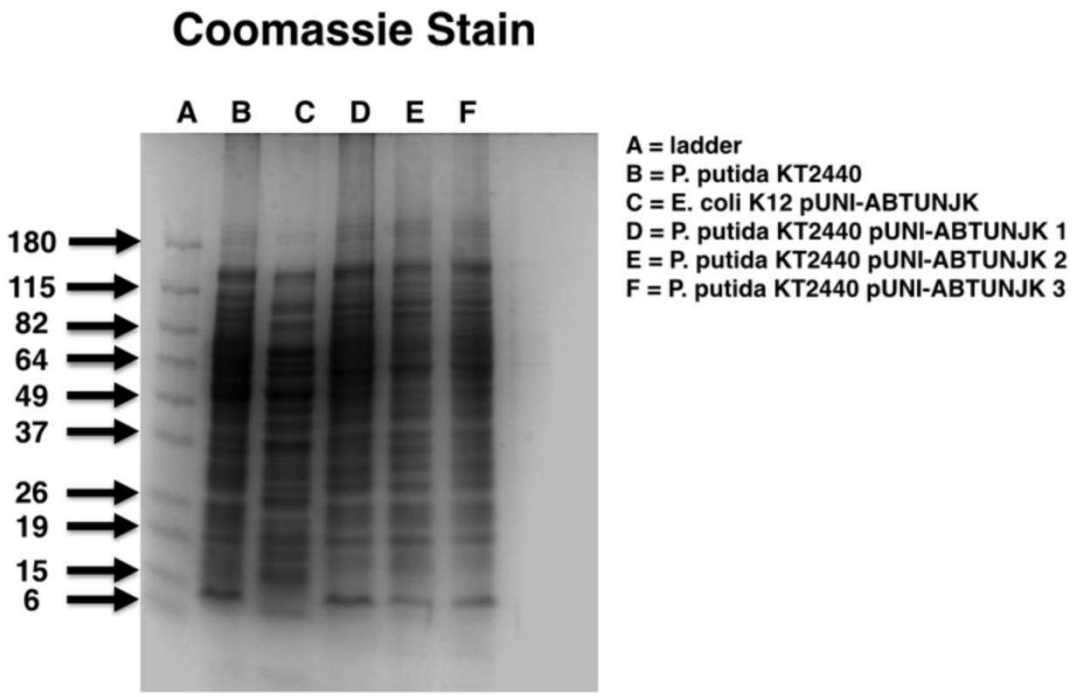
Early this week we designed primers for inserting the wild-type dcmA gene and its upstream region into DM4.

In order to confirm that our transformation of the pUNI-ABTUNJK into P. putida worked, we carried out a His-Tag In-Gel Stain of 3 samples of cultures grown overnight. While the bands in the sample lanes at first seem promising, we suspect that the stain was unspecific:

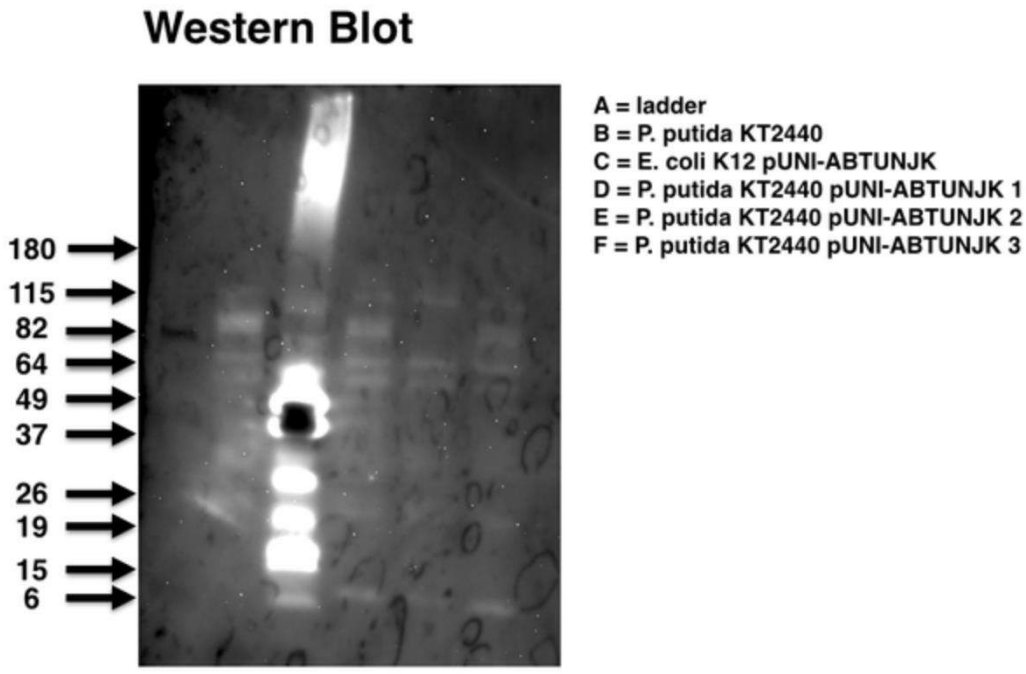


# Week 3 continued...

This was followed by Coomassie staining to detect the total amount of protein present. While a large amount of protein was observed in the sample lanes, no clear bands were detectable:




We also carried out a Western blot of the same 3 samples. Unfortunately, none of the sample lanes showed any chemiluminescence:



# Week 4

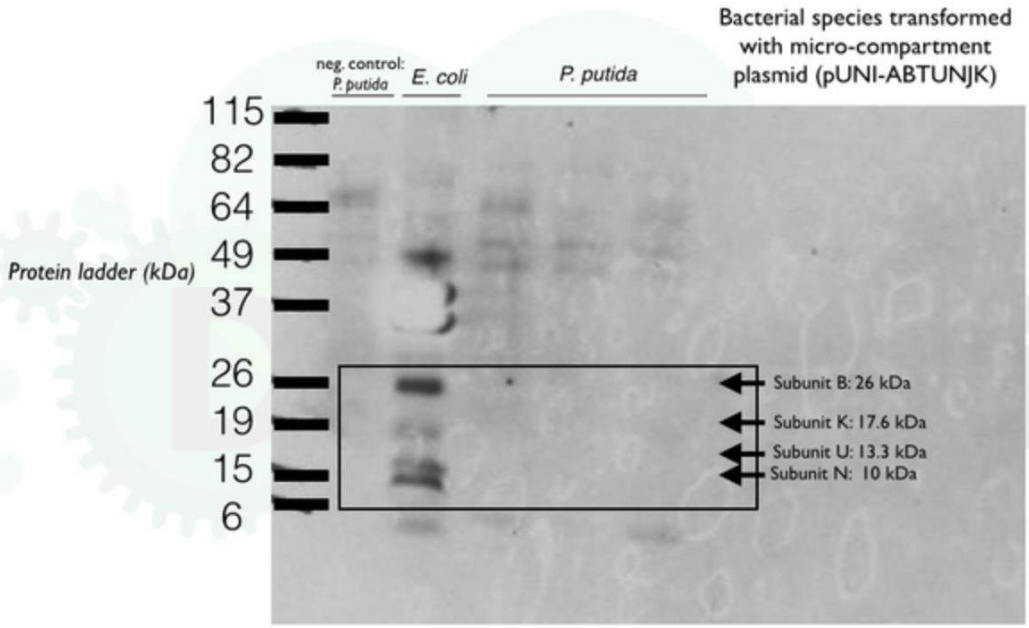
**Tasks completed:**

- Started building constructs for hypermutagenic PCR of dcmA
- Started building constructs for insertion of microcompartment into P. putida using a new vector
- Confirmed expression of microcompartments in E. coli

**Notebook:**

Since expression of ABTUNJK microcompartments did not work with the pUNI vector in P. putida, we have redesigned primers to insert ABTUNJK into the pSRKGm vector. Our first attempt at PCR-amplification of ABTUNJK and pSRKGm did not yield positive results, so we will repeat this next week with optimised settings.
Similarly, we have attempted to PCR-amplify the pCM66 vector in order to insert wild-type dcmA into it for hypermutagenic PCR. Unfortunately, this has not immediately yielded positive results so we have attempted another PCR-amplification again with slight variations of conditions, i.e. decreasing the annealing temperatures by 4°C. For the amplification of the pSRKGm vector, this has fortunately worked.

Finally, we have confirmed that last week's transformation of E. coli with the pUNI-ABTUNJK vector was successful by doing another western blot, as can be seen below:



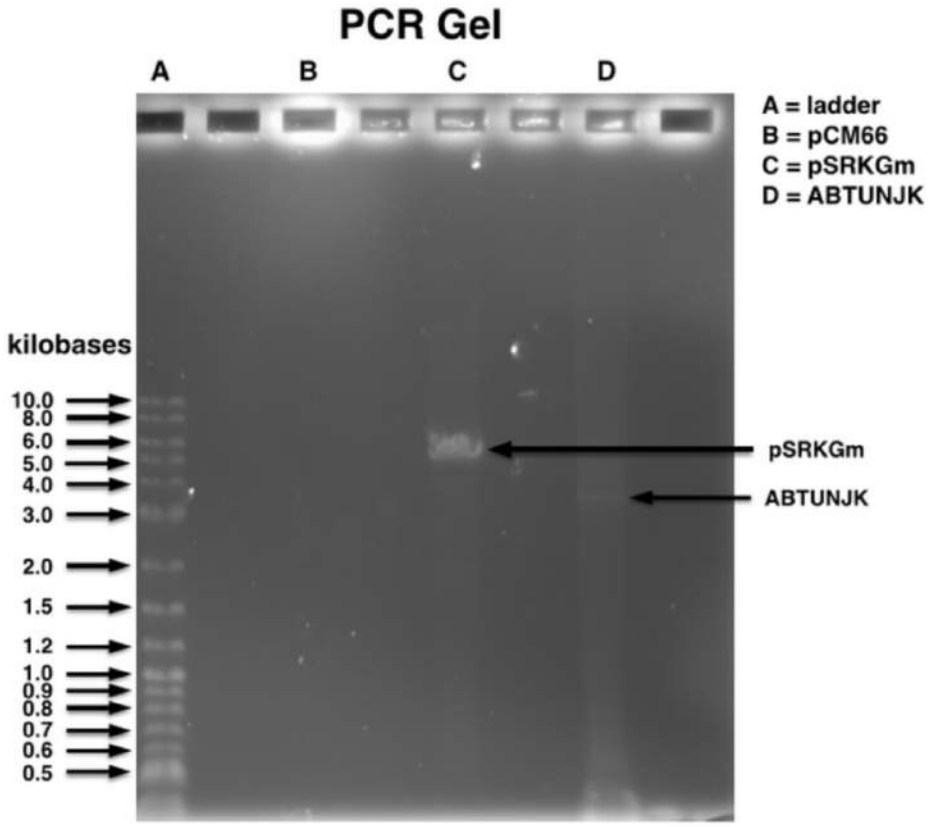
# Week 5

**Tasks completed:**

- PCR of pCM66
- PCR of pSRKGm
- PCR of ABTUNJK, followed by chaser PCR
- PCR of sfGFP
- PCR of dcmA
- Transformation of pRSFDuet into DH5α
- Transformation of sfGFP into DH5α
- Gibson assembly of dcmA vector into pCM66 backbone
- Gibson assembly of ABTUNJK vector into pSRKGm

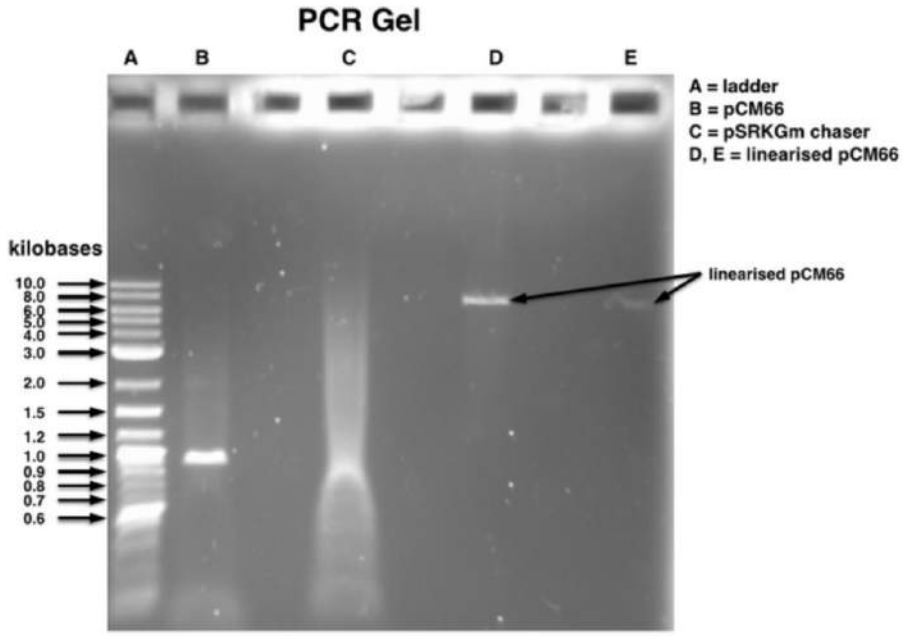
**Notebook:**

We successfully PCR amplified the pCM66 and pSRKGm backbones, as well as the ABTUNJK insert.

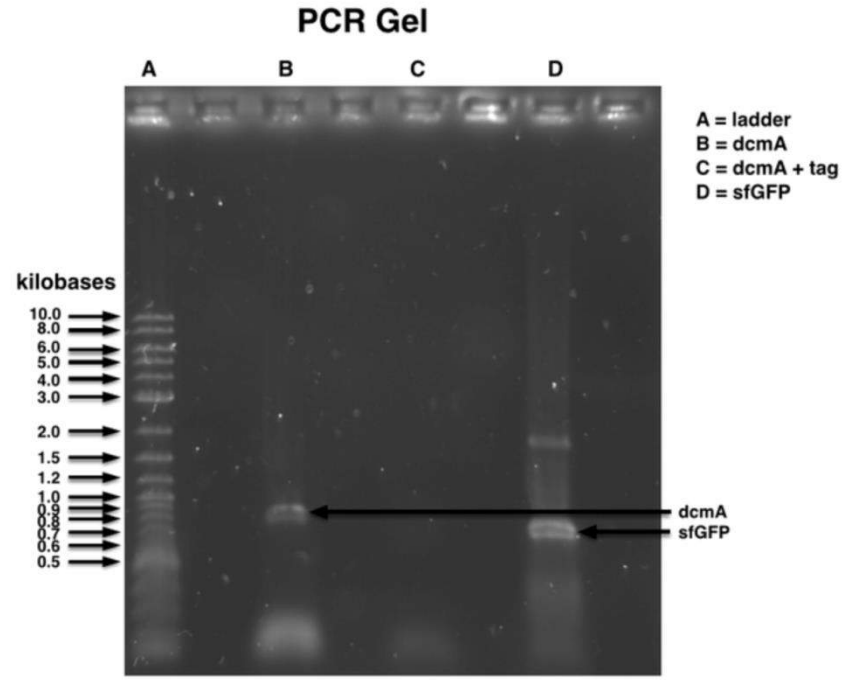


# Week 5 continued...

Since the concentration of ABTUNJK was very low, we followed this up with a chaser PCR:




We also PCR-amplified the dcmA insert and the sfGFP that we will use to visualise expression. Unfortunately, the dcmA with the microcompartment tag did not work, so conditions will be optimised for repeating this next week.




We also carried out two transformations: pRSFDuet and sfGFP both into a sample of DH5α E. coli cells.
Finally, we started assembling our constructs, using Gibson assembly to insert dcmA into pCM66 and to insert ABTUNJK into pSRKGm.

# Weeks 6 & 7

**Tasks completed:**

- Transformations and sequencing of Gibson products
- PCR of microcompartment-tagged sfGFP
- PCR to fuse dcmA and sfGFP
- Restriction and ligation of dcmA-sfGFP into pRSFDuet
- Gibson of ABTUNJK into pSRKGm
- Gibson of microcompartment-tagged sfGFP into pME6010
- Restriction and ligation of micro-compartment-tagged dcmA into pRSFDuet

We have successfully amplified dcmA and sfGFP by PCR with overlapping primers to fuse the two coding regions together with appropriate Restriction site upstream of dcmA (NcoI) and downstream of sfGFP (BamHI).
This allowed us to do a restriction digest on this fragment and the pRSFDuet vector. After the digest, we run the DNA on a gel and extracted the fragments using the standard protocol for DNA-gel extraction.
Finally, we set up a ligation overnight at 16°C and transformed the ligation product the next day into DH5alpha.

We took 6 colonies and made liquid cultures, mini prepped these the next day and sent these off for sequencing with 2 samples showing the expected sequence and correct gene fusion:


In addition to this, we have tried to assemble the microcompartment subunits into pSRKGm a second time, but this failed again. However, the Gibson of microtagged sfGFP into pMOxon2 was successful after doing a colony-PCR and sending two mini prepped samples off for sequencing.

# Weeks 8 & 9

**Tasks completed:**

- Fluorescence microscopy on microcompartment-tagged sfGFP and microcompartments as well as dcmA-GFP
- Restriction and ligation of dcmA into pRSFDuet

We tried a PCR on dcmA a second time in order to get the correct restriction sites-overhangs for ligation into pRSFDuet. This time, the PCR and ligation was successful after transformation, colony PCR and sequencing.

In addition to this, we have done fluorescence microscopy on both microtagged sfGFP with and without the microcompartment itself as well as on the dcmA-sfGFP fusion to see whether we can express dcmA in E.coli. We prepared our cells by transforming the constructs mentioned above into E.coli, followed by growing them in liquid cultures overnight.
In the morning we did 1 in 200 dilutions of the samples, added the inducer and antibiotics. The following amounts of inducer were added:
IPTG (for dcmA-sfGFP in pRSFDuet): 0, 10, 50 and 100 uM concentrations
ATC (for microtagged sfGFP in pOXON2): 0, 10, 100 and 500 ng/mL.

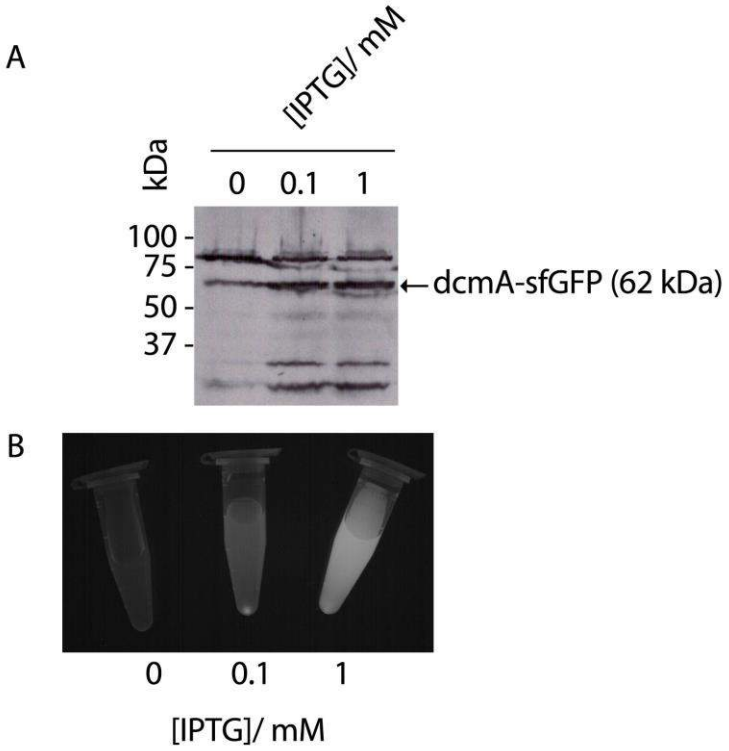
As shown on our results page, we did see dcmA being expressed in the cell, however we found brighter spots on the poles in some of the cells, indicating protein aggregation.

Interestingly, for our double-transformed E.coli with both plasmids (microtagged sfGFP and ABTUNJK), we saw localised and defined spots of higher fluorescence densities in some cells, indicating that the targeting mechanism of the N-terminal tag works successfully.

# Weeks 10 & 11

These last two weeks were dedicated to get our parts into the standard Biobrick vector pSB1C3 for submission. We used PCR with primers designed for 20-bp overhangs, Gibson assembly (as described above) and colony PCR in order to achieve this. We have managed to get microtagged sfGFP and microtagged dcmA into the standard vector.

It was shown that the dcmA-sfGFP construct was expressed in *E. coli*. A western blot was performed on whole cell lysates. A GFP primary antibody was used and a horseradish peroxidise secondary was conjugated to this primary. Thermo Scientific Pierce enhanced chemilumescent (ECL) Western Blotting Substrate was used according to the manufacturers description. The reaction was allowed to proceed 10 seconds and the substrate was subsequently removed. After 20 minutes photographic film was used to develop the image over a 5 minute period. The resulting western blot is given below.

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(A) A western blot showing the 62 kDa band for dcmA-sfGFP. It appears that the promoter is somewhat 'leaky' as even at 0 mM of IPTG there still appears to be expression of the construct. The heavier band may be the construct with an additional sfGFP molecule dimerised to it. The smaller bands may be explained by free sfGFP.

(B) Showing the expression of GFP in *E. coli* under blue LED light in live cells. Clearly expression increases with increasing concentration of IPTG.